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**DESIGN, SYNTHESIS AND TESTING OF
NOVEL ANTIMALARIAL COMPOUNDS**

by

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13. ABSTRACT The purpose of this project was to enhance the effectiveness of the class of chemicals known as isoquinolines against malaria, a disease causing over one million deaths each year. Many drugs are available to combat the parasite responsible for these deaths, but resistance has been recorded to nearly all of them. There is therefore a need for new antimalarial drugs against which the parasite is not resistant. As a contribution to the development of a new drug, this project involved modification of a lead compound, with known antimalarial properties, to enhance its effectiveness against the disease. The lead compound was a potent folate antagonist, a widely used class of antimalarial with significant resistance problems. Recent research had suggested that the lead compound acted against the disease through a distinct, unknown mechanism, in addition to that of folate antagonism. Six target compounds were designed to increase the activity by this unknown antimalarial mechanism, while removing activity via the folate mechanism. These novel target compounds were 1,7-disubstituted isoquinolines. The design included use of a 3D computer-generated pharmacophore model, which described the structural requirements for maximum antimalarial activity by the unidentified mechanism. Reduction of the folate antagonist activity relied upon published data describing the structural requirements for folate antagonism. The antimalarial activity of the newly designed compounds was thus expected to be mediated by the unknown mechanism. As such, the compounds should be effective even against parasite resistant to folate antagonists. Toxicity and synthesis issues were considered when the compounds were designed. A seven step synthesis was designed by which each of the six target compounds were prepared. A significant proportion of the project involved execution of these syntheses in the laboratory. Once prepared, the target compounds were submitted to the Walter Reed Army Institute of Research for <i>in vitro</i> antimalarial testing. The antimalarial data showed that the compounds possessed significant antimalarial activity and that the folate antagonist mechanism had been partially, if not completely, removed.				
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Abstract

The purpose of this project was to enhance the effectiveness of the class of chemicals known as isoquinolines against malaria, a disease causing over one million deaths each year. Many drugs are available to combat the parasite responsible for these deaths, but resistance has been recorded to nearly all of them. There is therefore a need for new antimalarial drugs against which the parasite is not resistant. As a contribution to the development of a new drug, this project involved modification of a lead compound, with known antimalarial properties, to enhance its effectiveness against the disease. The lead compound was a potent folate antagonist, a widely used class of antimalarial with significant resistance problems. Recent research had suggested that the lead compound acted against the disease through a distinct, unknown mechanism, in addition to that of folate antagonism. Six target compounds were designed to increase the activity by this unknown antimalarial mechanism, while removing activity via the folate mechanism. These novel target compounds were 1,7-disubstituted isoquinolines. The design included use of a 3D computer-generated pharmacophore model, which described the structural requirements for maximum antimalarial activity by the unidentified mechanism. Reduction of the folate antagonist activity relied upon published data describing the structural requirements for folate antagonism. The antimalarial activity of the newly designed compounds was thus expected to be mediated by the unknown mechanism. As such, the compounds should be effective even against parasite resistant to folate antagonists. Toxicity and synthesis issues were considered when the compounds were designed. A seven step synthesis was designed by which each of the six target compounds were prepared. A significant proportion of the project involved execution of these syntheses in the laboratory. Once prepared, the target compounds were submitted to the Walter Reed Army Institute of Research for *in vitro* antimalarial testing. The antimalarial data showed that the compounds possessed significant antimalarial activity and that the folate antagonist mechanism had been partially, if not completely, removed.

malaria; synthesis; drug

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Introduction¹

The World Health Organization estimates that there are 300 to 500 million new cases of malaria each year, directly resulting in over one million deaths annually. Over 40 percent of the world's population lives in regions suffering from the tropical disease, which has become a significant cause of death in developing nations. Malaria has taken its toll on the U.S. military serving in these countries as recently as September 2003, when 36% of Marines and Sailors deployed in Liberia contracted the disease, over half of whom had to be evacuated.² In humans, malaria can be caused by four species of protozoan parasites: *Plasmodium (P.) falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The most serious conditions arise from infection by *P. falciparum* and may include heart, lung, kidney and brain problems or death. Resistance to many currently used anti-malarial drugs has been documented in *P. falciparum* and *P. vivax*.³

Several different anti-malarial drugs are currently available. Quinine was the first effective antimalarial agent and is largely now replaced by compounds with improved activity. Effectiveness of new drugs is often reported relative to quinine activity. Despite the number of available drugs, there is a demand for new anti-malarial compounds because of growing resistance occurring to the existing drugs. Once the parasites are resistant to the mode of action of a certain compound, this new strain is potentially resistant to other drugs employing that same mechanism. However, drugs acting by a different mode of action will still be effective against this new strain. Therefore, one strategy to combat malaria is to deploy new treatments more quickly than the parasite can adapt and develop resistance.

¹ Work described in the introduction section was conducted by others and reported prior to commencement of this project.

² COL Wilbur Milhous, USA, Personal Communication.

³ Bloland, P. B. *Drug Resistance in Malaria*; World Health Organization: Switzerland, 2001; pp 1-27.

Antimalarial drug development consists of modifying a starting compound, called a lead, which has known antimalarial effectiveness. Previous research at USNA has explored a class of compounds called chalcones. Although the specific method of action is unknown, chalcones are known to act against malaria in a different manner than of the current drugs. The most effective chalcone found at USNA so far is shown in Figure 1. The antimalarial activity is quantified by the IC_{50} , the amount of compound necessary to inhibit *in vitro* parasite growth by fifty percent.

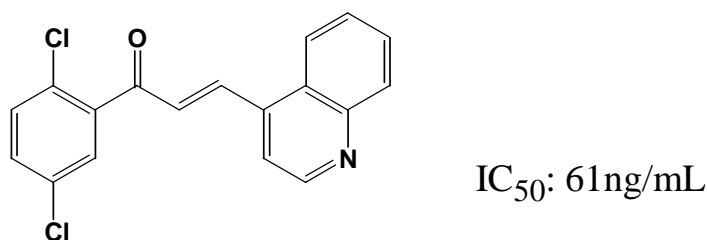


Figure 1 – Most Active Chalcone Found in USNA Research.

Low IC_{50} values are desired since lower IC_{50} s represent greater potency. The highest IC_{50} value acceptable in a molecule for it to be tested more extensively is 30ng/mL. The IC_{50} of the chalcone shown above is thus too high for it to be considered for further testing as a possible drug. Compounds with improved antimalarial activity are therefore needed.

The structures and the corresponding antimalarial activities from the chalcone research were compiled at the Walter Reed Army Institute of Research to create a computer-generated pharmacophore model. This model is a three-dimensional schematic describing the requirements of structure and chemical functional groups for maximum antimalarial activity in compounds acting by the chalcone mechanism. Figure 2 shows the four different structural features that comprise the chalcone pharmacophore: an aliphatic hydrophobic region (blue), two aromatic regions (blue and grey), and a hydrogen bond donor (purple).

Though the pharmacophore model is generated from antimalarial activities of actual compounds, it can be used to predict the activity of new compounds before they are even

synthesized. The degree to which the new structure matches the pharmacophore determines the predicted antimalarial activity of the novel compound.

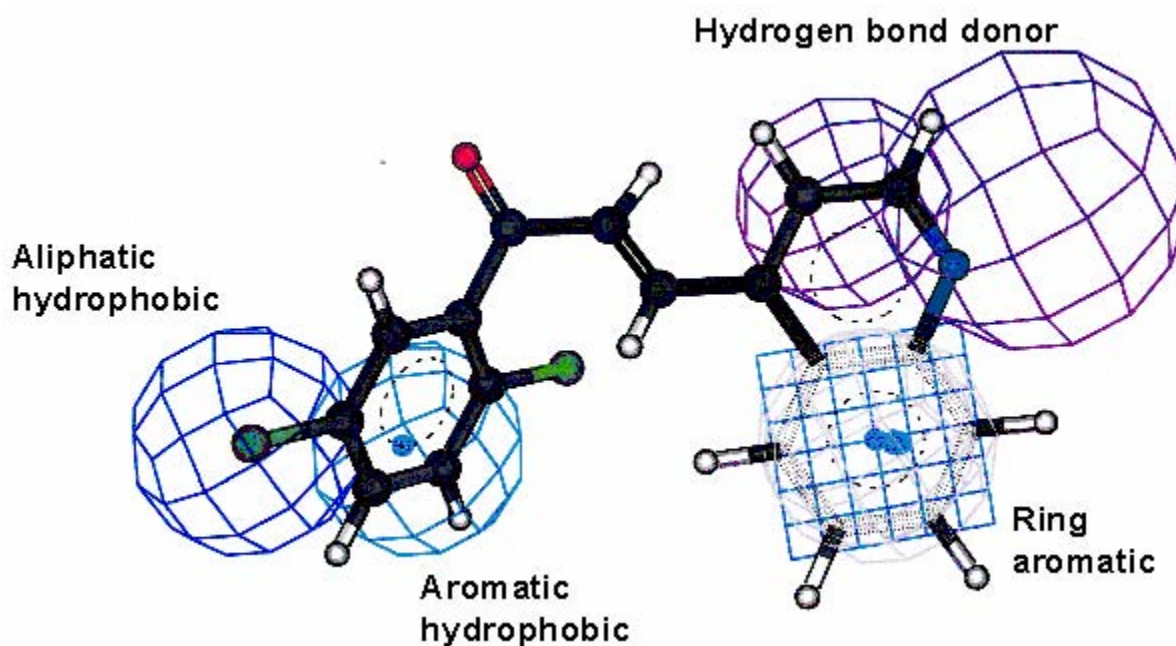


Figure 2 – Chalcone from Figure 1 Overlapping Pharmacophore Model.

Figure 2 also shows how the chalcone in Figure 1 overlaps with the pharmacophore, the overlap is good except for a missing hydrogen bond donor (purple region). It is expected that fulfilling this requirement will improve the antimalarial activity.

To this end, the extensive collection of proprietary compounds at Walter Reed Army Institute of Research was screened for similarity to the pharmacophore. Of several compounds that emerged from this screening process, one in particular appeared to possess significant overlap with the chalcone pharmacophore, and is shown in Figure 3.

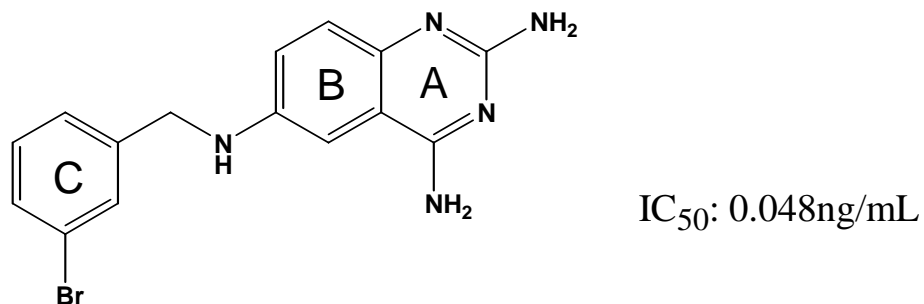


Figure 3 – The Lead Compound: 2,4-Diamino-6-[(3-bromobenzyl)amino]quinazoline.

Figure 4 shows how this compound overlaps with all four elements of the pharmacophore, and should be compared with Figure 2 where only three elements are satisfied.

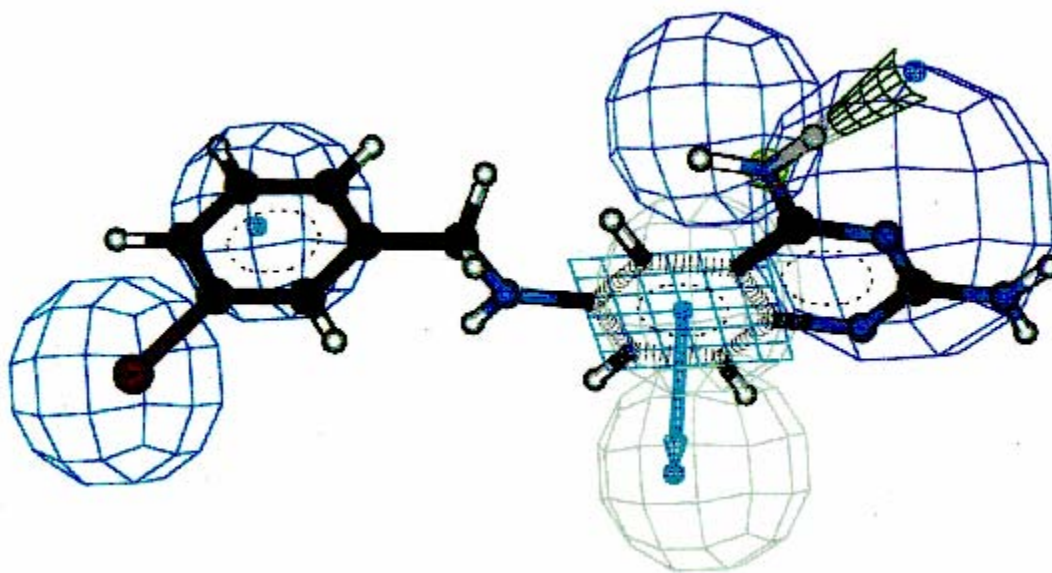


Figure 4 – The Lead Compound Overlapping the Pharmacophore Model.

The compound shown in Figure 3 was therefore selected as the new lead for this project. This project sought to design new molecules based upon its structure with the expectation that they would be better antimalarial compounds than those synthesized at USNA to date.

This lead compound was already known as a potent folate antagonist, a class of antimalarial compounds. In fact, the reason this compound was in the WRAIR chemical library was that it had been part of their folate antagonist studies.

Since the 1970's the plasmodial enzyme dihydrofolate reductase (DHFR) has been recognized as a viable target for antimalarial drugs because it is essential for parasite life and distinct from the vertebrate version of DHFR.⁴ Compounds that inhibit the parasite DHFR are known as folate antagonists, or antifolates. DHFR is required for the reduction of dihydrofolate into tetrahydrofolate, which is needed in the synthesis of deoxyribonucleic acid, DNA. Compounds that can block DHFR have a similar structure to folate (see Figure 6), occupying the enzyme's active site and preventing the binding of dihydrofolate. Without DHFR function, the organism cannot replicate its genetic material and reproduction ceases.⁵ Research of folate antagonists has produced drugs such as proguanil, chlorproguanil-dapsone, pyrimethamine, and trimethoprim, as well as numerous promising leads for new drugs.

Many folate antagonists have shown activity in treating malaria but have limited effectiveness due to development of drug resistance by the parasites. General interest in folate antagonists exists today due to their effectiveness in Sub-Saharan Africa, low relative cost, and ability to combine effectively with other antimalarials.⁶

Derivatives of 2,4-diaminoquinazoline can act as folate antagonists because their structure is similar to folate. Adding substituents to the 6-position of the quinazoline, exemplified in Figure 5, enhances antimalarial properties of the compound.

⁴ Roth, B.; Cheng, C. C. *Prog. Med. Chem.* **1982**, 19, 275, 269-331.

⁵ Parenti, M. D.; Pacchioni S.; Ferrari, A. M.; Rastelli, G. *J. Med. Chem.* **2004**, 47, 4258-4267.

⁶ *Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Directions in Drug Discovery*; Rosenthal, P. J., Ed.; Humana Press: Totowa, NJ, 2001, pp 173-190.

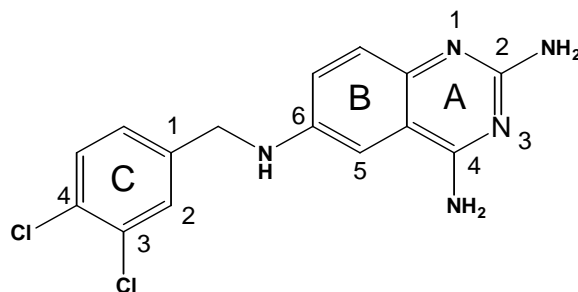


Figure 5 – 2,4-Diamino-6-[(3,4-dichlorobenzyl)amino]quinazoline.

Combining structural elements that individually contribute to antimalarial activity can optimize the overall antimalarial activity of a compound. For example, in 1972, Dr. Edward Elslager (working at the Parke, Davis and Company) began with 2,4-diamino-6-[(3,4-dichlorobenzyl)amino]-quinazoline, known to be a potent antimalarial compound. Systematic changes to just one part of the structure were made. By comparing the effectiveness of many such modified compounds in treating mice infected with the *P. berghei* species of malaria, the parts of the compound responsible for antimalarial activity were determined. Potency was reported in two ways, as an SD_{90} value and as a quinine equivalent, Q. The SD_{90} value represents the dose required for the suppression of *P. berghei* in 90% of the mice, compared to the control mice which did not receive any treatment. A lower dose required for suppression equates to higher potency of that drug. Results were also assessed by calculating the ratio of the compound's SD_{90} and the SD_{90} of quinine hydrochloride, producing a quinine equivalence for the compound.

Forty-six 6-substituted 2,4-diaminoquinazolines were synthesized by the Elslager group. Most of these compounds differed in their structures at the 2-, 3-, and/or 4- positions of ring C. Out of these forty-six, twenty-seven were found to be more active than quinine in mice, even against Plasmodium strains resistant to pyrimethamine and other antimalarial drugs. In monkeys, the results were just as strong, showing nineteen compounds with activity, seven of which were

curative. Although drug intolerance was not evident at the small doses required for the antimalarial tests, higher doses in early toxicological tests produced weight loss in proportion to the amount of compound given.

Over the course of twenty-two published papers, from 1972 to 1984, the Elslager group explored this promising new class of compounds. Small changes in the molecular structure had significant effects on the antimalarial activity of a compound. When ring C was replaced with various heterocyclic rings, the compounds showed problems with toxicity in mice and low antimalarial activity in chicks.⁷ It was found that changes made to the linker between the two ring systems, specifically incorporation of alkyl groups, resulted in tremendous increases in antimalarial activity.⁸ Cross-resistance, a type of resistance developed from exposure to similar drugs, appeared against a group of the compounds.⁹ Elslager's group found that while most linkers containing sulfur conferred no antimalarial benefits,¹⁰ sulfonyl linkers increased antimalarial activity but resistance was induced quickly.¹¹

Overall, Elslager found that 2,4-diaminoquinazolines were a potent class of antimalarials, but were ultimately rejected for further antimalarial research since resistance to them developed quickly. Elslager eventually abandoned work on the entire class of compounds. These studies clearly indicated that it would be critical to significantly reduce, or ideally remove, the folate antagonist activity from the lead compound of this project because of the resistance issues. Removal of the folate antagonism properties from the lead were aimed at resulting in compounds

⁷ Elslager, E. F.; Clarke, J.; Werbel, L. M.; Worth, D. F.; Davoll, J. *J. Med. Chem.* **1972**, 15, 827-836.

⁸ Elslager, E. F.; Bird, O. D.; Clarke, J.; Perricone, S. C.; Worth, D. F. *J. Med. Chem.* **1972**, 15, 1138-1146.

⁹ Worth, D. F.; Johnson, J.; Elslager, E. F.; Werbel, L. M. *J. Med. Chem.* **1978**, 21, 331-337.

¹⁰ Elslager, E. F.; Davoll, J.; Jacob P.; Johnson, A. M.; Johnson, J.; Werbel, L. M. *J. Med. Chem.* **1978**, 21, 639-643.

¹¹ Elslager, E. F.; Hutt, M. P.; Jacob, P.; Johnson, J.; Temporelli, B.; Werbel, L. M.; Worth, D. F.; Rane, L. *J. Med. Chem.* **1979**, 22, 1247-1257.

acting by solely the chalcone mechanism, and therefore devoid of the pre-existing resistance problems.

The fact that the lead overlapped well with the chalcone pharmacophore suggested that it acts by the chalcone mechanism in addition to folate antagonism. The exact biological target for the chalcone mechanism is currently unknown, but antimalarial testing shows the mechanism to be distinct from that of folate antagonism: Tests were performed against three parasite strains, including one resistant to folate antagonists. Any compound producing successful results against two strains but not against the resistant strain is most likely a folate antagonist. Because chalcones possess similar activities against all three strains, it was concluded that the chalcone mechanism was distinct from folate antagonism.

Design of the Target Compounds¹²

This project sought to design six novel compounds, based upon the lead compound 2,4-diaminoquinazoline, expected to possess good chalcone-like activity against malaria. As outlined in the introduction, another design criterion is reduction in antifolate activity. Additionally, since there appear to be toxicity issues with the lead, low toxicity must be designed into new compounds. These compounds should have the potential for further progression towards the development of a new antimalarial drug. Moreover, the data obtained from such a series of potent compounds acting exclusively by the chalcone-like mechanism should be useful towards refining the pharmacophore model.

Reduction in folate antagonism was performed by locating and removing the chemical groups responsible for this activity. In folate antagonists, the ring A contributes to antimalarial activity because it resembles folate, seen in Figure 6. This similarity allows folate antagonists to act like folate and bind to the DHFR enzyme, thereby inhibiting an essential biological reaction in the parasite causing malaria. It was expected that changes made to the structure of ring A would decrease folate antagonist activity. The proposed modifications were based on knowledge of the role of specific atoms involved in the folate antagonist mechanism and on pharmacophore testing.

¹² The design of this research project was conducted by the author. Extensive consultation of the primary literature and discussion with Dr. Gutteridge were involved. Dr. Bhattacharjee conducted the computer modeling requested by the author, namely generating the predicted IC₅₀ and toxicity data reported in Figures 10 and 11. Dr. Gerena conducted the antimalarial testing requested by the author, namely generating the IC₅₀ data reported in Figure 9.

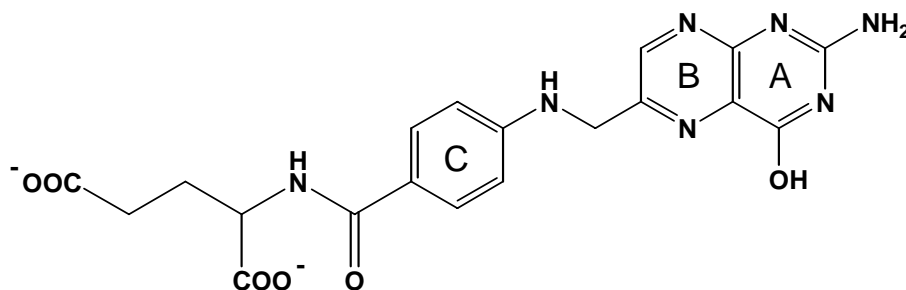


Figure 6 – Folate. Note the A/B ring systems are similar to those of the lead compound

Because folate antagonists function through their similar structure to folate, altering the structure to reduce resemblance to folate should remove antifolate activity. The parts of the structure important for folate antagonism were reported in two studies. One of the studies identified the sites on the quinazoline which interact attractively with DHFR. Among these sites are the N-1 and 2-NH₂ groups.¹³ It was therefore deemed desirable to remove these two nitrogen atoms. In the second study, again the 2-NH₂ group was shown to be important for the binding to the DHFR enzyme, suggesting its removal would inhibit the ability of the compound to bind to DHFR.¹⁴

Fortunately, the effect of altering the A ring on the chalcone-like antimalarial activity could be tested using the pharmacophore model. To this end, four potential variants of the A ring were proposed (Figure 7). The remainder of the structure was kept identical to that of the lead. Computation of the predicted IC₅₀'s (using the pharmacophore model) and likelihood of toxicity, were requested by the author. The computer-generated prediction of toxicity results from thirty toxicity-predictor tests. The reported data is the number of toxicity tests failed out of 30 (an acceptable score is less than 7).

¹³ Roth, B.; Cheng, C. C. *Prog. Med. Chem.* **1982**, 19, 275, 269-331.

¹⁴ Ghose, A. K.; Crippen, G. M. *J. Med. Chem.* **1984**, 27, 901-914.

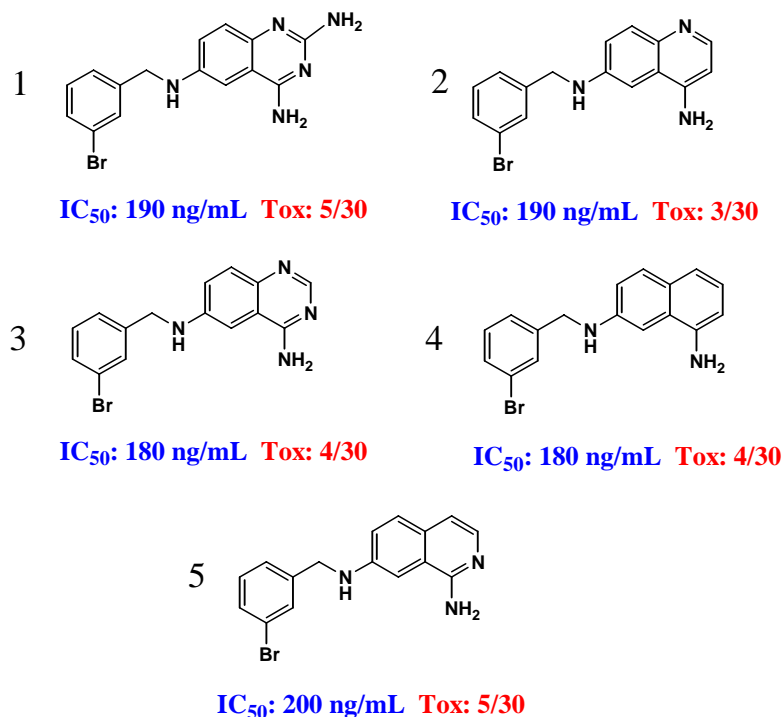


Figure 7 – Compounds with Different Nitrogen-placements on Ring A, with Predicted IC₅₀ and Toxicity. Neither IC₅₀ nor Toxicity score pointed at a particular compound.

The predicted IC₅₀s of the structures showed little variation. The same was true for the toxicity results. The arrangement of atoms in ring A of structure #5, Figure 7, was chosen for incorporation into the newly designed compounds for two reasons. First, the literature showed that the arrangement of the nitrogen atoms in #5 minimizes folate antagonist activity. Second, the remaining NH₂ group fulfills all four requirements of the pharmacophore, including the aliphatic hydrophobic group, (as the lead compound did in Figure 4) so chalcone-like activity was expected to be maximized.

Because rings B and C overlapped well with the pharmacophore, it was decided to retain them in the newly designed compounds. However, it was seen in the Elslager papers that the chemical groups, or substituents, bonded to ring C contributed significantly to their activity against malaria. Modification of the lead at these substituents on ring C were therefore explored. To find which substituents had the most potential for improving antimalarial activity, the author

chose 14 compounds in stock in the chemical library at the Walter Reed Army Institute of Research which had a similar structure to the lead compound. The only difference between the structures of these 14 compounds were in the groups connected to ring C. Therefore, disparities in the antimalarial activities could be attributed solely to the corresponding groups on ring C. The *in vitro* testing of these compounds, requested by the author, was performed on three strains of malaria causing parasite: D6, TM91-C235, and W2. The D6 and W2 parasite strains are not resistant to the effects of folate antagonists, while the TM91-C235 strain is resistant to many drugs, including folate antagonists. The selected compounds and their resulting IC₅₀'s are shown in Figure 8. Note the typically ten-fold lower activity of these compounds against the resistance strain compared to the non-resistant strain, highlighting the fact that the lead, and structurally related compounds, suffer drug-resistance problems. Six variants of the C ring A were chosen from these results in addition to those of the Elslager group, completing six newly-designed structures (shown in Figure 10).

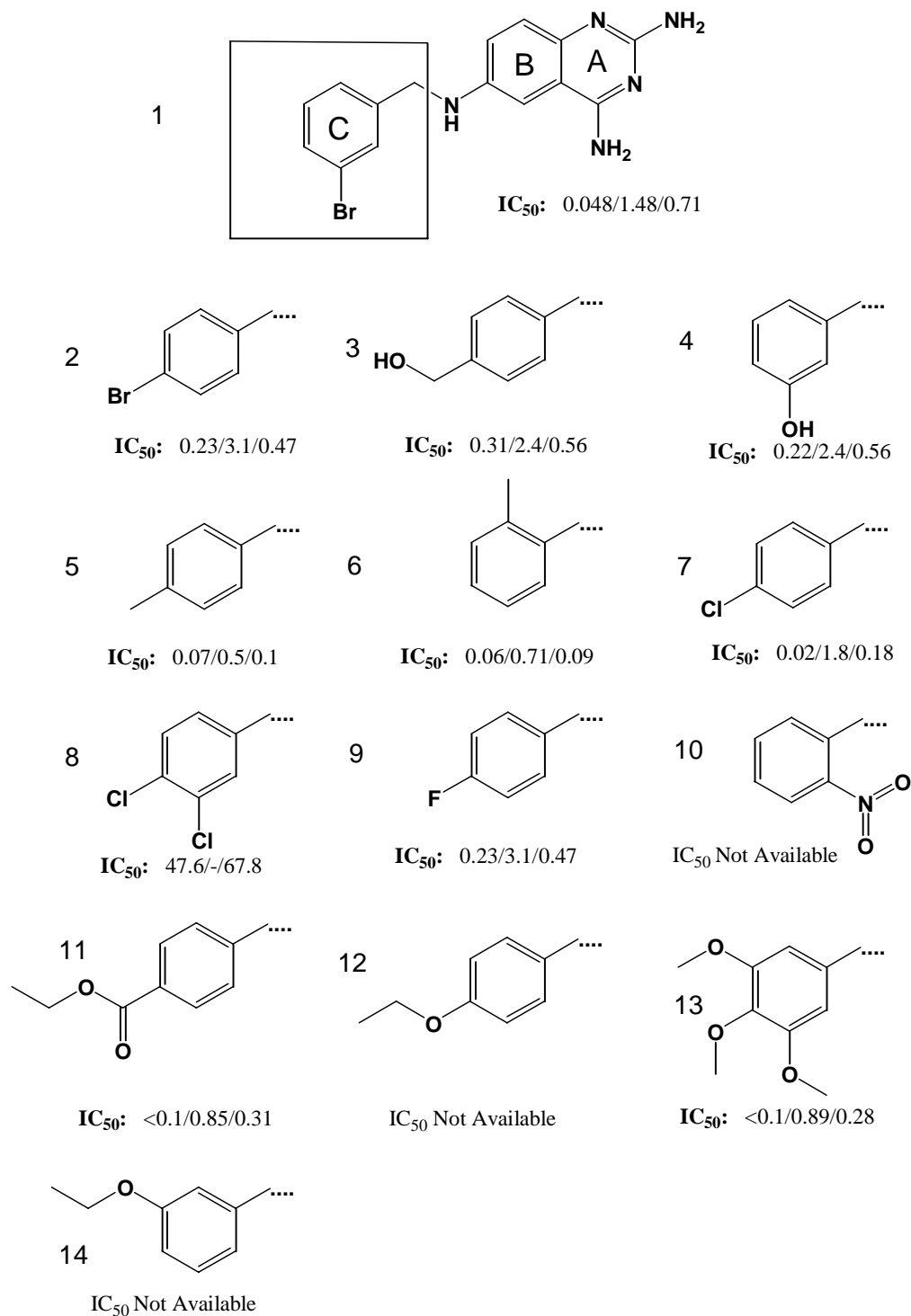


Figure 8 – Compounds with Different Ring C Substituents.
Antimalarial IC₅₀s against three *P. falciparum* strains (D6/TM91-C235/W2) in ng/mL were determined at WRAIR.

A third region of the lead whose variation was explored was the linker between the rings.

Six variations in the linker, largely suggested by the work from the Elslager group, were modeled in the same computer prediction programs as the ring A set.

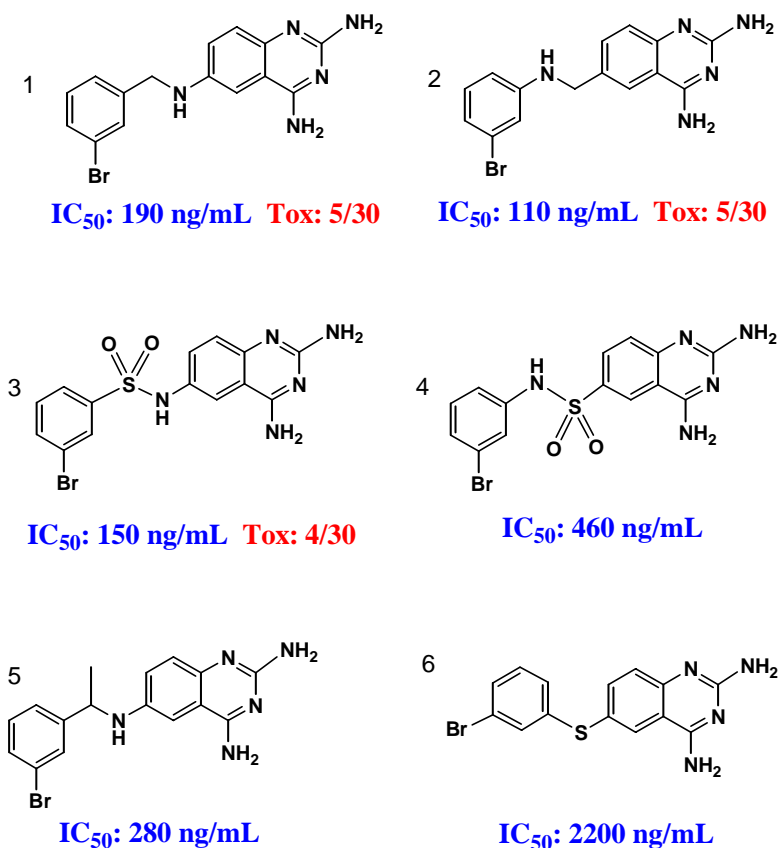


Figure 9 – Compounds with Different Linkers.
 Compounds with IC₅₀ above 200 ng/mL were rejected.

The predicted IC₅₀'s varied greatly for the different linker structures. This is probably due to differences in the angle between the two ring systems with the different linkers, leading in some cases to poor matching to the pharmacophore model. Three compounds with very high predicted IC₅₀'s were rejected. In terms of toxicity scores, the remaining three were roughly equivalent. Structure #2 of this set was disregarded because of synthesis difficulty and Structure #3 was rejected for problems with combining the chosen A ring with this linker.

After combining the chosen ring C substituents, the optimal linker and the favored substitution pattern of ring A, six novel compounds were proposed for synthesis. The ultimate goal is to have a minimum of twelve milligrams of each of the six final compounds for submission to WRAIR for antimalarial testing.

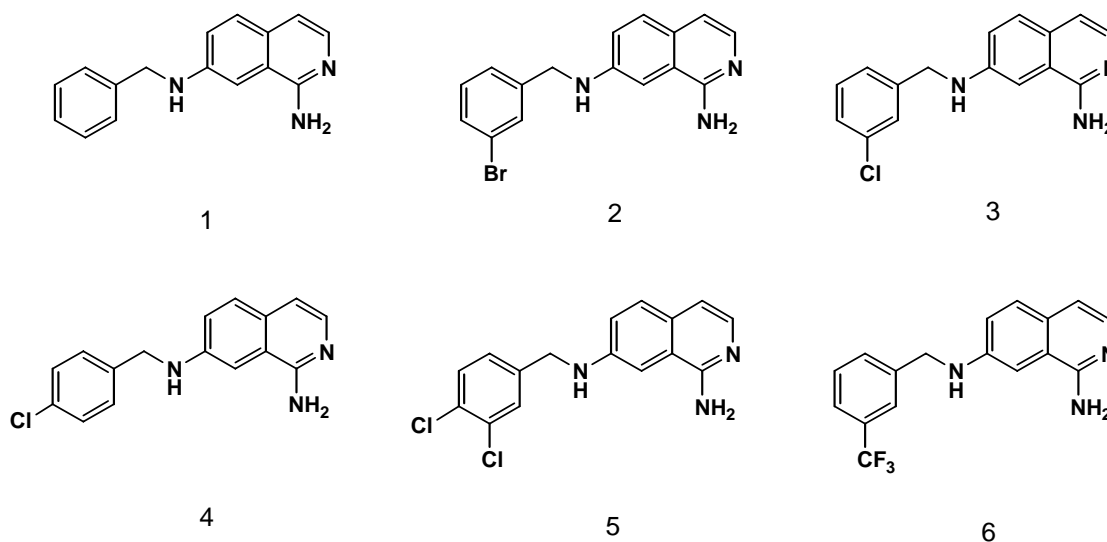


Figure 10 – The Six Target Compounds.

These compounds have never been synthesized before and are designed to possess activity against malaria.

Predicted antimalarial activities of these compounds were obtained using the pharmacophore model. Target 1 does not fulfill all of the requirements of the pharmacophore model, leading to the very low activity prediction in Figure 11. Targets 2 through 6 were predicted to have considerable antimalarial activity.

Compound	IC ₅₀ (ng/mL)	Compound	IC ₅₀ (ng/mL)
Target 1	7800	Target 4	210
Target 2	200	Target 5	170
Target 3	210	Target 6	190

Figure 11 – Predicted IC₅₀s of the Target Compounds against the W2 Strain of *P. falciparum*.

Design of Synthesis

The synthetic scheme shown in Figure 12 shows the series of chemical reactions and solvents proposed to produce the six novel compounds designed in this project. Each arrow represents the reaction from one compound to another. The synthetic scheme was designed so that the individual reactions to produce the six different compounds occurred as late as possible in the plan. This saved the project time and chemicals. There was literature precedence for these reactions: all but the last reaction are described in the literature, albeit with very little experimental detail. Similar reactions to the last have been performed by others, but the exact reaction and product proposed are novel. The ultimate goal was to have a minimum of twelve milligrams of each of the six final compounds for submission to WRAIR for antimalarial testing.

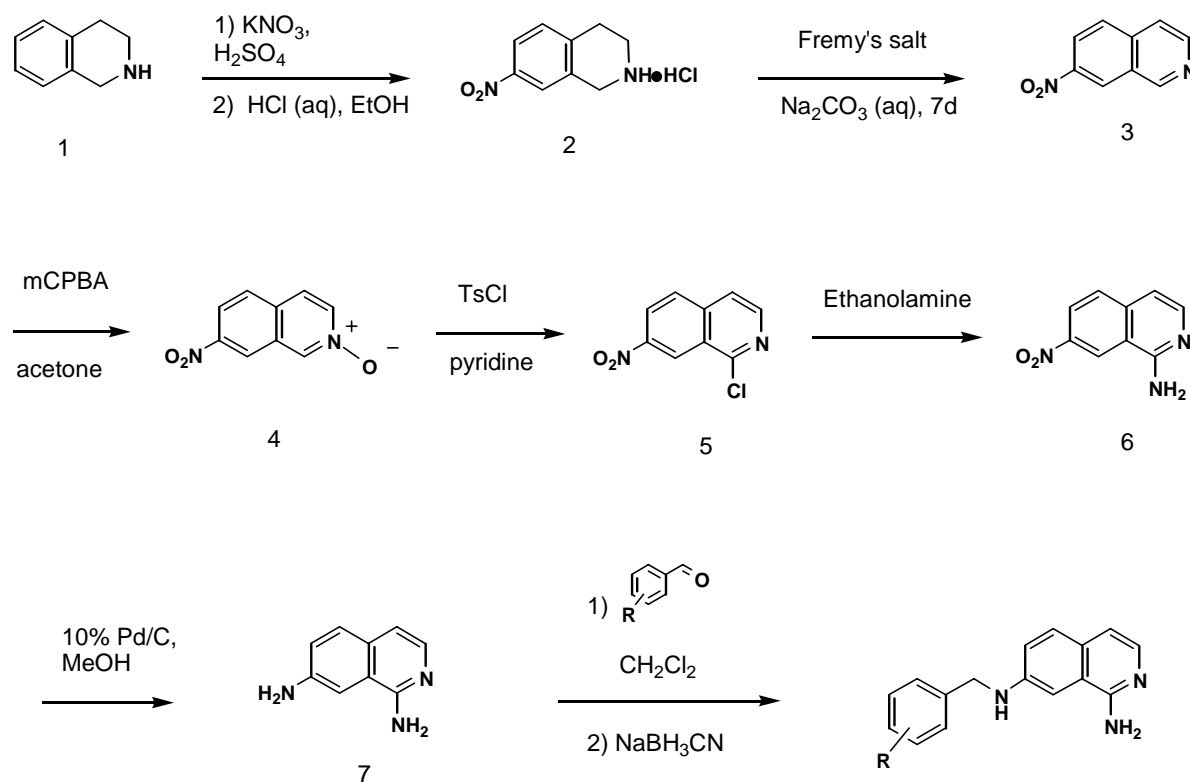


Figure 12 – Synthetic Scheme for the Six Novel Compounds of Figure 10.
The R group represents the six possible chemical groups in the six target compounds.
Note that the first seven steps were identical for the synthesis of the six different compounds.

Experimental

Synthesis of the six novel compounds began with the commercially available starting material **Compound 1**, 1,2,3,4-tetrahydroisoquinoline. The reactions to produce **Targets 1** through **6** (Figure 10) via **Compounds 2** through **7** (Figure 12) are described in this section. Typically, reactions were performed by placing the starting material, a reactant and a solvent in a flask with a magnetic stirrer. Most reactions were stirred at room temperature for a few hours, but some were conducted at cold or hot temperatures, and some required shorter or longer reaction times, up to many days. Each reaction was performed several times. The first time a reaction was carried out, it was conducted with a small amount of starting material to make sure that the reaction proceeded as expected. Normally the reaction scale was then stepped three-fold. This scale-up was repeated until the reaction scale was large enough to yield a usable amount of product. At the beginning of the synthesis this means tens of grams of starting material, towards the end of the synthesis the largest reactions were typically run with about a tenth of a gram of starting material. Once the reactions were complete, purification was normally required to isolate the desired compound from contaminants. Purification techniques included extraction, which separates based on solubility, and column chromatography, which separates according to polarity. The larger the reaction scale, the harder work the purification: A typical small-scale column chromatography takes about 2 hours and uses about one liter of solvent, on a larger scale it can take upwards of 6 hours and 10 liters of solvent. Structural confirmation of the product compounds was performed mainly through Proton Nuclear Magnetic Resonance (^1H NMR), and is described in the Characterization section. This technique is almost definitive, requires only a few milligrams of compound and takes only a few minutes to perform.

To produce **Compounds 2** through **6**, extensive use was made of two reports.^{15,16} Since both were patents, the descriptions of these procedures were minimal and much experimentation was required to learn how to perform the reactions. Generally, the reactions worked well, but there were complications. For instance, the first reaction if conducted as described would have yielded a different compound than the one described. Fortunately a paper reporting how to obtain the desired compound (**Compound 2**) was consulted before experimentation began.¹⁷ Later in the synthesis, during the step to prepare **Compound 4**, several reagents, expected to yield the same product, seemed to produce different products. Determination of which reaction, if any of these, was giving the desired product was exacerbated by the difficulty of characterizing the *N*-oxide (Compound 4).

To produce crystalline compounds, which were easier to handle than the oily solids of **Targets 1** through **6**, a reaction with hydrochloric acid was employed to produce hydrogen chloride salts.

A detailed technical description of the laboratory work is reported in Appendix A.

¹⁵ Ajao, J. F.; Bird, C. W. *J. Heterocycl. Chem.* **1985**, 22, 329-31.

¹⁶ Zhu, B.; Su, T.; Zhaozhong, J.; Scarborough, R. M.; Song, Y. World Patent WO 2000071507, 2000.

¹⁷ Potter, M. D.; Taylor, E. P. *J. Chem. Soc.* **1953**, 1320-1321.

Characterization

To characterize the compounds produced, a technique known as nuclear magnetic resonance (NMR) was employed. This technique takes advantage of the small natural magnetic field created by each atom's spinning protons. The orientation of this magnetic field which is normally random is aligned along a specific axis when an extremely large magnetic field is created by the NMR instrument. Whether the nucleus is aligned in a low or high energy state is dependent upon the strength of the external magnetic field. After a specific magnetic field strength is applied, the nucleus enters the high energy state. The necessary strength of the field is dependent on the type of nucleus being observed. In this project, the ^1H nucleus was used. When the ^1H nucleus returns to the lower energy state, it releases measurable radio-frequency energy. After the resulting pattern is put through a Fourier transformation the NMR spectrum is obtained.¹⁸

There are several qualities of the nucleus that affect the NMR spectrum. One of these is the nuclear shielding due to surrounding electrons, which lessen the strength of the magnetic field on a particular nucleus. Nuclei that are near electron-withdrawing groups will experience more of the magnetic field than if they had more electrons surrounding them. In this situation, the peak is farther left, or downfield, than it would be without the electron-withdrawing group nearby.

Another factor that affects the NMR spectrum is the surrounding nuclei. Identical nuclei, those that are in the same chemical environment, produce identical peak signals. Comparing integrals of the peaks can be used to find how many identical nuclei are represented by each peak. Nearby nuclei of different chemical environments lead to a splitting of the peak signals

¹⁸ Huffman, P. D. Trident Scholar Paper, United States Naval Academy, Annapolis, MD, 2001.

from singlet peaks to doublets, triplets, etc. The position, integral and splitting of each peak in the NMR spectrum are used to identify nuclei and the overall structure.

In the ^1H NMR spectrum (Figure 14) for the starting material (**Compound 1**), 1,2,3,4-tetrahydroisoquinoline, the total integral of the peaks around 7ppm is approximately 4 (3.946), these corresponding to the four ^1H nuclei attached to the aromatic ring of Compound 1. Also notice how the remaining seven ^1H nuclei are represented by the four peaks from 1.7 to 4.0ppm, whose total integral is approximately 7 ($2.005 + 2.000 + 2.051 + 1.234$).

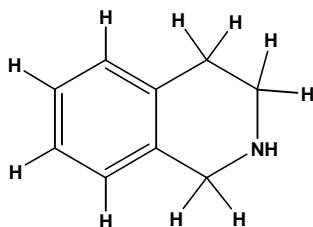


Figure 13 – Starting Material with Hydrogen Atoms (^1H) Shown.

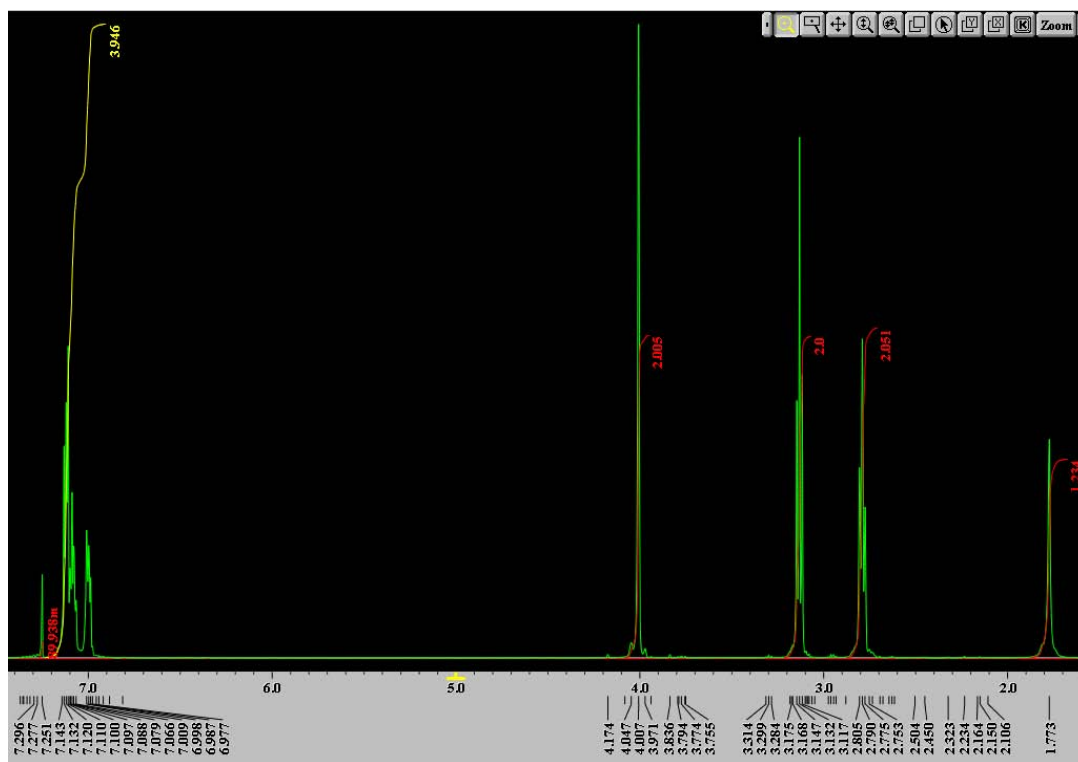


Figure 14 – ^1H NMR of the Starting Material, Compound 1.

The NMR spectrum of each compound was carefully analyzed to ensure the expected number of peaks, peak positions, and splitting patterns were observed. In most cases this was sufficient to ensure the isolated compound had the desired structure.

A few compounds required further analysis to support their structures. The *N*-oxide (**Compound 4**) structure was supported by a correct $M+1$ peak in Maldi-MS, ^{13}C NMR peak shifts, and an infrared absorbance around $1200\text{-}1300\text{cm}^{-1}$. The *N*-oxide structure was also supported by the fact that the two different reactions, one using *m*-chloroperbenzoic acid in acetone and the other using hydrogen peroxide, seemed to produce the same product, as shown by ^1H NMR.

The NMR spectra for all the compounds prepared are included in the Appendix B.

Antimalarial Results¹⁹

The six synthesized compounds of this project were submitted to the Walter Reed Army Institute of Research in April 2006. Since these tests were too complex for anyone but an expert to run, the author simply observed these tests being performed. The compounds were tested at ten concentrations, ranging from 12500 ng/mL to 24 ng/mL and the data was used by the experts to obtain an IC₅₀. The antimalarial results for Target Compounds 1, 2 and 3 are shown in Figure 15. Compound were tested against three strains of *P. falciparum*, W2, D6 and TM91-C235. The resistance properties of these strains are shown in Figure 16.

Compound	IC ₅₀ (ng/mL) <i>D6 Strain</i>	IC ₅₀ (ng/mL) <i>W2 Strain</i>	IC ₅₀ (ng/mL) <i>TM91-C235 Strain</i>
Best Chalcone	71.4	60.50	Not Determined
Lead Compound	0.71	0.048	1.48
Target 1	450.80	68.36	156.47
Target 2	144.14	26.00	63.55
Target 3	148.66	26.24	73.06

Figure 15 – Antimalarial Testing Results of Target Compounds 1, 2 and 3.
Also shown are the results of the lead compound and the most active chalcone synthesized at USNA to date.

¹⁹ The antimalarial *in vitro* tests were performed by Dr. Lucia Gerena at the Walter Reed Army Institute of Research.

	D6	W2	TM91-C235
Resistance	Mefloquine Halofantrine	Chloroquine Pyrimethamine	Mefloquine Chloroquine Quinine Folate Antagonists
Susceptibility	Chloroquine Pyrimethamine	Mefloquine	

Figure 16 – Resistances and Susceptibilities of *P. falciparum* Strains Tested.
The D6 strain does not have complete resistance to mefloquine and halofantrine, only reduced susceptibility.

At WRAIR, each compound was tested alongside chloroquine and mefloquine. The IC_{50} s of these drugs determined during this project are reported in Figure 17.

Antimalarial Drug	IC_{50} (ng/mL) <i>D6 Strain</i>	IC_{50} (ng/mL) <i>W2 Strain</i>	IC_{50} (ng/mL) <i>TM91-C235 Strain</i>
Chloroquine	4.16	124.09	21.2
Mefloquine	7.9	2.46	15.08

Figure 17 – Antimalarial Data of Chloroquine and Mefloquine Determined Experimentally in this Project.

Although the target compounds were less active than chloroquine and mefloquine, the low IC_{50} s showed the compounds to be worthy of further investigation. The results showed that Targets 1, 2 and 3 had comparable antimalarial activity to the most active chalcone synthesized at the United States Naval Academy to date.

Comparison of the data for the TM91-C235 strain with that for the other strains suggests that the folate antagonist activity originally possessed by the lead compound has been partially, if not completely, removed. This removal of the folate antagonist mechanism was also the most likely reason for the target compounds having less activity than the lead compound. Further investigation of the difference in activities against the D6 and W2 strains will be undertaken, since these results may suggest possible cross-resistance with chloroquine.

The results obtained were very close to the pharmacophore predicted IC_{50} values, seen in Figure 11, suggesting that the pharmacophore used in this project was accurate.

Assuming that future analysis shows the antimalarial activity is due to solely the chalcone-like mechanism, the data will be incorporated into the pharmacophore, further enhancing its accuracy.

Conclusion

The novel antimalarial compounds synthesized in this project will aid antimalarial research in at least two ways. Foremost, the antimalarial testing results will facilitate researchers in designing other novel compounds, as previous studies assisted in the design of this project. The compounds proved to have worthy IC_{50} 's and upon further analysis they could undergo the next phase of antimalarial testing, namely an assessment of their ability to treat a mouse infected with malaria. Testing is required to determine if the folate antagonist activity was removed. This testing can be done with an assay that evaluates the ability of a compound to bind DHFR.

Secondly, the project is a contribution to the area of organic synthesis because the six targets compounds have never been synthesized before. The experimental details enabling their synthesis has been carefully documented, leaving an easy-to-follow record for other researchers to make similar compounds. This record, currently in laboratory notebooks, contains all of the necessary procedures, data, and observations to perform the reactions again, will be published in a paper.

Significant obstacles were overcome in executing the synthesis using the available published record. A few reactions, such as the step leading to **Compound 3** and the reaction producing the *N*-oxide (**Compound 4**), were fully explored with a variety of reactants and solvents. In the case of the former reaction, it was found that using either Fremy's Salt or iodine yielded similar results. With the latter, several reagents, expected to yield the same product, seemed to produce different products.

In addition to these benefits to antimalarial research, these compounds may assist research working on other diseases. The remainder of the six target compounds synthesized for

this project are kept in the chemical database at the Walter Reed Army Institute of Research and are available for any researcher to request testing against other ailments such as cancer or *trypanosomiasis*.

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Appendix A – Technical Procedure

Compound 2

Tetrahydroisoquinoline (**Compound 1**) (50mL, 53g, 0.398mol) was added slowly to sulfuric acid (200mL), maintaining the temperature around 5°C, but never reaching above 20°C. Potassium nitrate (43.14g, 0.428mol) was added to the stirring mixture, maintaining the temperature below 5°C. The reaction turned pale orange and was allowed to stir at room temperature for 24 hours. The resulting dark brown oil was poured onto ice, then ammonium hydroxide was added until the pH reached 10. The mixture was extracted into chloroform (1.8L), washed with saturated sodium chloride solution (2 x 100mL), dried over magnesium sulfate and filtered. After concentration to dryness, the resulting dark orange oil was dissolved in ethanol (500mL). A yellow precipitate formed when concentrated hydrochloric acid (43.5mL) was added. This hydrogen chloride salt was purified by recrystallization using methanol (900mL). White crystals were obtained after vacuum filtration (22.4g, 0.105mmol, 26%). The reaction was completed 4 other times beginning with quantities ranging from 3g to 20g, with yields as high as 36%.

Compound 3

To the isoquinoline salt (**Compound 2**) (2.16g, 10mmol) was added a solution of disodium nitrosodisulfonic acid, also known as Frey's Salt, (30.1g, 112mmol) in 4% sodium carbonate solution (0.453L). The dark purple reaction was allowed to stir for 7 days. It turned brown within one day and showed a yellow precipitate by the end of this time. The mixture was extracted into chloroform. Recrystallization was attempted using petroleum ether, as described in

the literature,¹⁷ and using an ether/ethyl acetate mixture but the solid did not dissolve well. The solid was therefore purified by column chromatography (using alumina oxide and hexane:ethyl acetate = 1:9) to give **Compound 3** (0.532g, 3.0mmol, 30%). This reaction was performed 4 other times beginning with starting material amounts ranging from 0.7g to 3.59g.

An alternative method¹⁷ to obtain **Compound 3** was explored. The isoquinoline salt (3.00g, 14mmol) and potassium acetate (6.01g, 61mmol) were dissolved in ethanol (51.4mL). While the mixture was refluxing, iodine (7.16g, 28mmol) in ethanol (66.4mL) was added, and the reaction continued refluxing for 18 hours. The resulting dark brown mixture was filtered from a yellow precipitate. About half the solvent was removed under pressure and precipitate appeared again. Concentrated aqueous HCl (4.26mL) and water (17.17mL) was added to the filtrate and the mixture became clear. The remaining ethanol was removed under pressure, water (13mL) was added and the mixture was filtered. The mixture was washed with ether. To the aqueous layer (at pH 1), 20% sodium hydroxide solution (20mL) was added until the pH reached 7. The cloudy mixture was filtered to obtain a light orange solid (0.95g). The solid was purified by column chromatography (using alumina oxide and hexane:ethyl acetate = 2:3) as performed in other literature¹⁶ to give **Compound 3** (0.593g, 24%). This reaction procedure was followed from the literature and was repeated 3 other times beginning with quantities ranging from 0.7g to 16g.

Compound 4

Compound 3 (1.7g, 9.8mmol) was dissolved in acetone (70mL) and *meta*-chloroperoxybenzoic acid (4.04g, 16mmol at 70-75%) was added giving a clear orange solution. The reaction was allowed to stir for 21 hours, during which time a yellow solid precipitated. The

mixture was dried under pressure to give a bright yellow solid. This solid was dissolved in dichloromethane (300mL), washed with saturated sodium bicarbonate solution (300mL) and then saturated sodium chloride solution. The organic layer was dried over magnesium sulfate, filtered and concentrated to dryness yielding **Compound 4**, a bright yellow solid (3.01g). This reaction was performed six times before at smaller scales ranging from 70mg to 1.33g. This reaction was performed according to the literature.¹⁵

Compound 5

Pyridine (70mL) was added to **Compound 4** (3.01g, 9.79mmol *N*-oxide), which did not dissolve until tosyl chloride (2.24g, 11.7mmol) was added, giving an orange solution. The reaction was stirred at room temperature for 2 hours. The mixture was concentrated to dryness, yielding a dark brown syrup. This sample of **Compound 5** was used without further purification. This reaction was performed twice before at smaller starting amounts of 62.3mg and 423mg of the *N*-oxide.

Compound 6

Ethanolamine (150mL) was added to **Compound 5**, and the dark viscous mixture was stirred at room temperature for 18 hours. The mixture was diluted with dichloromethane (600mL) and the amber colored solution was washed with water (500mL). This washing was repeated with dichloromethane (200mL) and water (200mL). The dark orange aqueous layer was discarded while the bright yellow organic layer was washed with saturated sodium chloride solution (100mL) then dried over magnesium sulfate. The solution was concentrated to dryness, then partitioned between ethyl acetate (3 x 100mL) and 5% sodium hydroxide solution (100mL). The organic layer was washed with saturated ammonium chloride and 2N hydrochloric acid

(100mL) was added. The aqueous phase from this wash was treated with 20% sodium hydroxide to yield an orange precipitate of **Compound 6** which was recovered by filtration. The solid was purified by column chromatography (using alumina oxide and 1% methanol in ethyl acetate) to give **Compound 6** (280 mg, 1.48mmol, 15% yield from Compound 4).

Compound 7

Compound 6 (260mg, 1.38mmol) was dissolved in methanol (100mL), and 10% palladium on carbon (105mg) suspended in methanol (3mL) was added. The mixture was allowed to stir at room temperature and was successively evacuated and purged with nitrogen three times. The nitrogen gas was replaced with hydrogen gas and the reaction was left stirring for 15 hours. The mixture was filtered through celite and dried under pressure yielding a pale yellow solid (254mg, quantitative). This reaction was performed twice before at smaller scales (113mg and 146mg) with yields near 67% and 71%. This reaction was performed according to the literature.²⁰

Target 1

The amine (**Compound 7**) (88 mg, 0.55mmol) was dissolved in acetic acid (1.65mL), benzaldehyde (112μL, 1.10mmol) was added and the reaction allowed to stir at room temperature for 30 min. Sodium cyanoborohydride (1.65mL, 1M THF solution) was added and the reaction stirred for 3 hours. The mixture was poured into icewater, the pH adjusted to 11 by addition of sodium hydroxide, then the mixture extracted into ethyl acetate. Column chromatography (alumina, eluting with 5% methanol in ethyl acetate) afforded **Target 1**

²⁰ Shinkai, H.; Ito, T.; Yamada, H. World Patent WO 9948492, 1999.

(79.7mg, 0.32mmol, 58%). This reaction was performed according to the literature.²¹ Treatment with ethanolic HCl followed by evaporation yielded the hydrogen chloride salt (135.3mg).

Target 2

The amine (**Compound 7**) (86mg, 0.54mmol) was dissolved in acetic acid (1.62mL), 2-bromobenzaldehyde (126μL, 1.08mmol) was added and the reaction allowed to stir at room temperature for 30 min. Sodium cyanoborohydride (1.62mL, 1M THF solution) was added and the reaction stirred for 3 hours. The mixture was poured into icewater, the pH adjusted to 11 by addition of sodium hydroxide, then the mixture extracted into ethyl acetate. Column chromatography (alumina, eluting with 5% methanol in ethyl acetate) afforded **Target 2** (44.5mg, 0.136mmol, 25%). Treatment with ethanolic HCl followed by evaporation yielded the hydrogen chloride salt (55.9mg).

Target 3

The amine (**Compound 7**) (86mg, 0.54mmol) was dissolved in acetic acid (1.62mL), 2-chlorobenzaldehyde (123μL, 1.08mmol) was added and the reaction allowed to stir at room temperature for 30 min. Sodium cyanoborohydride (1.62mL, 1M THF solution) was added and the reaction stirred for 3 hours. The mixture was poured into icewater, the pH adjusted to 11 by addition of sodium hydroxide, then the mixture extracted into ethyl acetate. Column chromatography (alumina, eluting with 5% methanol in ethyl acetate) afforded **Target 3**. Treatment with ethanolic HCl followed by evaporation yielded the hydrogen chloride salt (84.5mg).

²¹ Gunzenhauser, S.; Balli, H. *Helv. Chim. Acta.* **1989**, 72, 1186-1198.

Target 4

The amine (**Compound 7**) (70.5mg, 0.443mmol) was dissolved in acetic acid (1.33mL), 3-chlorobenzaldehyde (129mg, 0.88mmol) was added and the reaction allowed to stir at room temperature for 30 min. Sodium cyanoborohydride (1.33 mL, 1M THF solution) was added and the reaction stirred for 3 hours. The mixture was poured into icewater, the pH adjusted to 11 by addition of sodium hydroxide, then the mixture extracted into ethyl acetate. Column chromatography (alumina, eluting with 5% methanol in ethyl acetate) afforded **Target 4** (93.4mg, 0.33mmol, 74%). Treatment with ethanolic HCl followed by evaporation yielded the hydrogen chloride salt (85.4mg).

Target 5

The amine (**Compound 7**) (72mg, 0.445mmol) was dissolved in acetic acid (1.37mL), 3,4-dichlorobenzaldehyde (159.3mg, 0.88mmol) was added and the reaction allowed to stir at room temperature for 30 min. Sodium cyanoborohydride (1.37 mL, 1M THF solution) was added and the reaction stirred for 3 hours. The mixture was poured into icewater, the pH adjusted to 11 by addition of sodium hydroxide, then the mixture extracted into ethyl acetate. Column chromatography (alumina, eluting with 5% methanol in ethyl acetate) afforded **Target 5** (93.9mg, 0.30mmol, 65%). Treatment with ethanolic HCl followed by evaporation yielded the hydrogen chloride salt (78.7mg).

Target 6

The amine (**Compound 7**) (70mg, 0.44mmol) was dissolved in acetic acid (1.32mL), 3-(trifluoromethyl)benzaldehyde (132 μ L, 0.88mmol) was added and the reaction allowed to stir at room temperature for 30 min. Sodium cyanoborohydride (1.32mL, 1M THF solution) was

added and the reaction stirred for 3 hours. The mixture was poured into icewater, the pH adjusted to 11 by addition of sodium hydroxide, then the mixture extracted into ethyl acetate. Column chromatography (alumina, eluting with 5% methanol in ethyl acetate) afforded **Target 6** (45.8mg, 0.144mmol, 33%). Treatment with ethanolic HCl followed by evaporation yielded the hydrogen chloride salt (51.9mg).

Appendix B – NMR Spectra

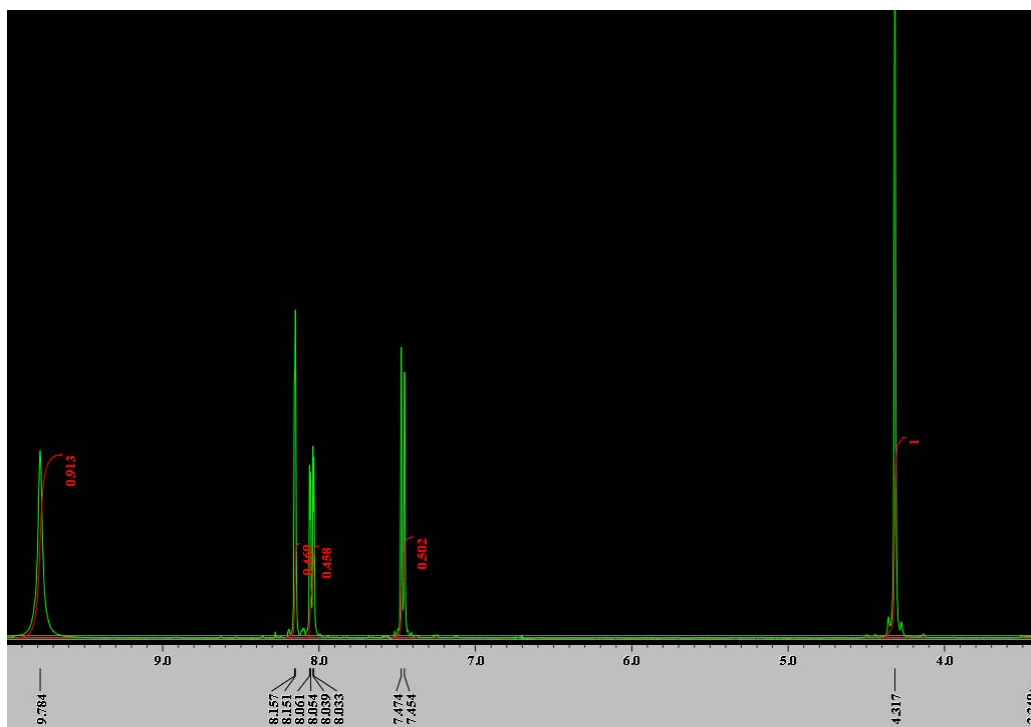


Figure 18 – ¹H NMR Spectra of Compound 2.

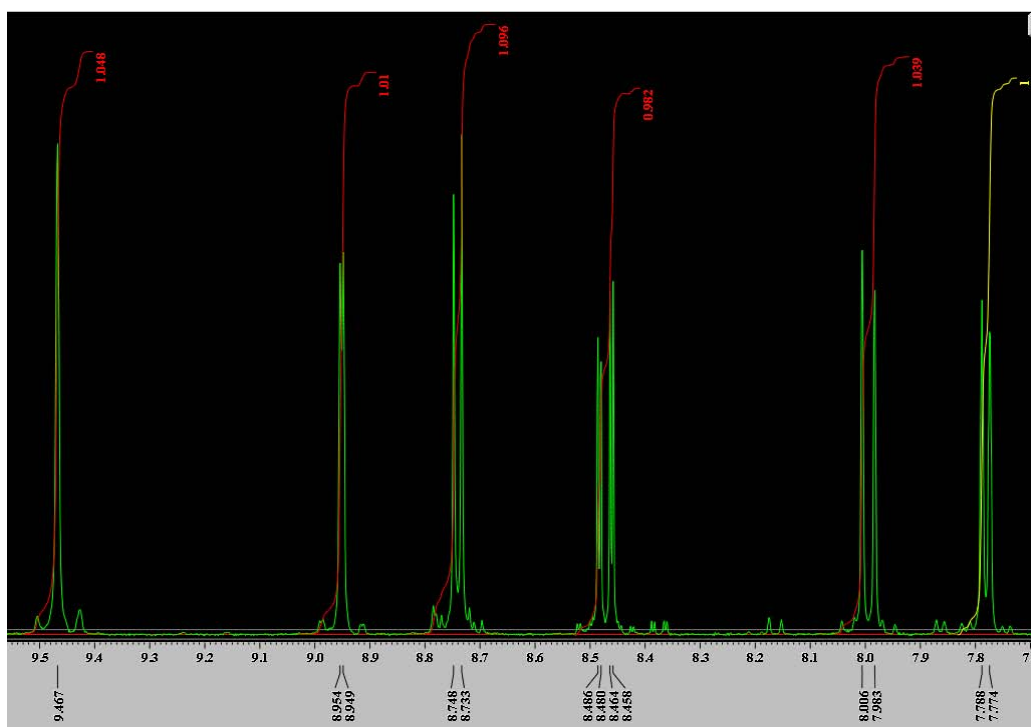


Figure 19 – ¹H NMR Spectra of Compound 3.

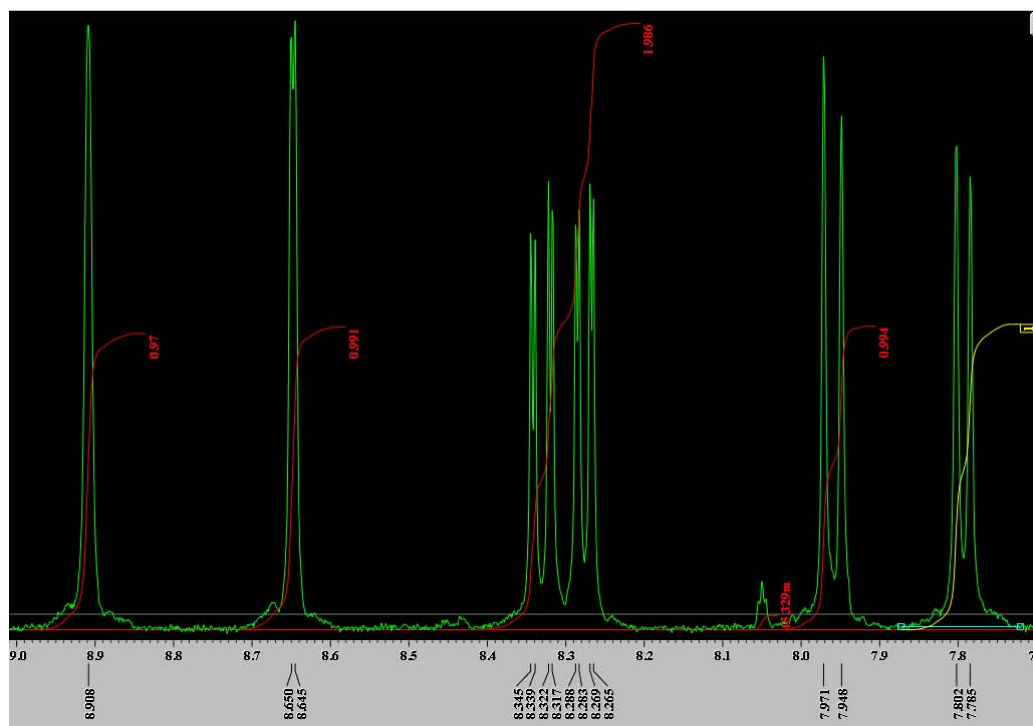


Figure 20 – ¹H NMR Spectra of Compound 4.

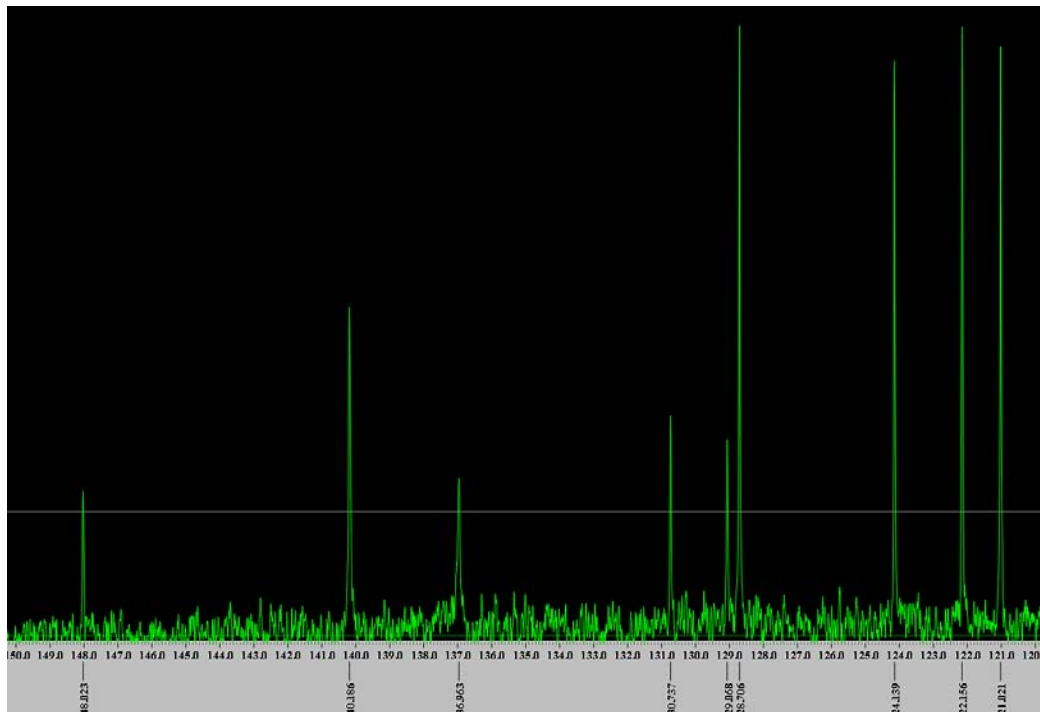
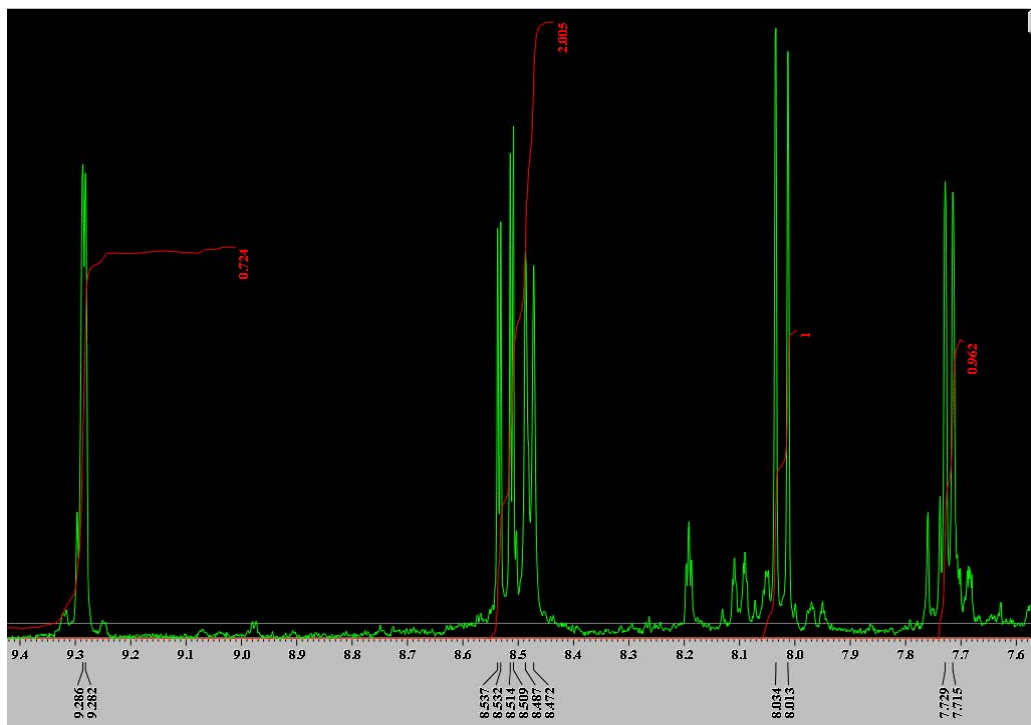
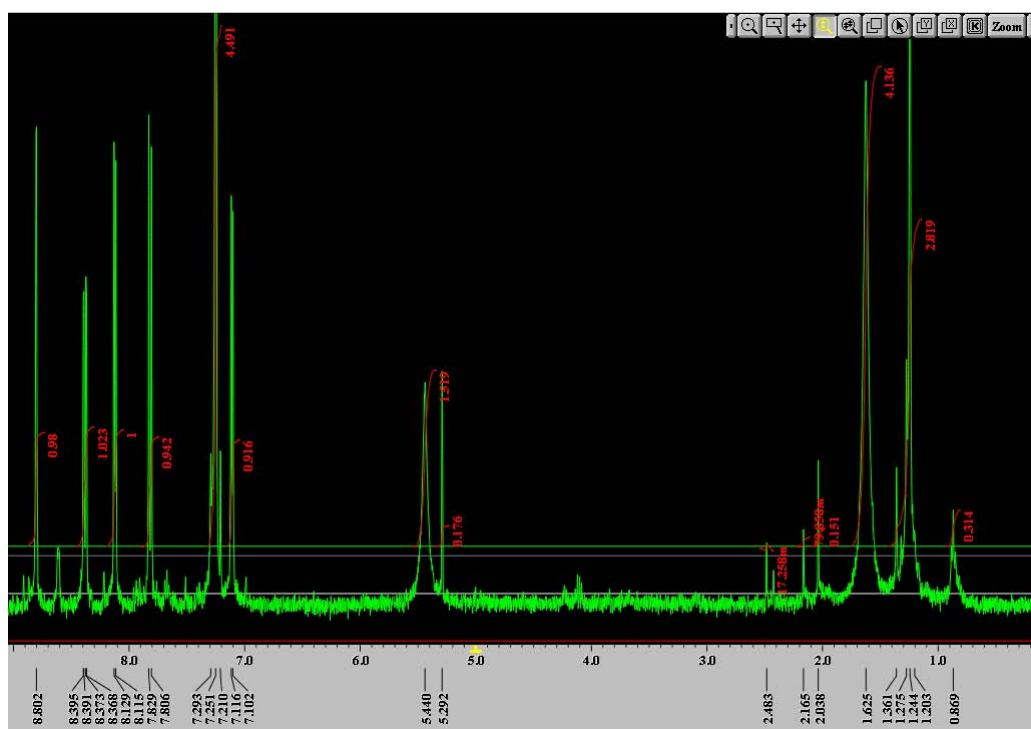


Figure 21 – ¹³C NMR Spectra of Compound 4.

Figure 22 – ¹H NMR Spectra of Compound 5.Figure 23 – ¹H NMR Spectra of Compound 6.

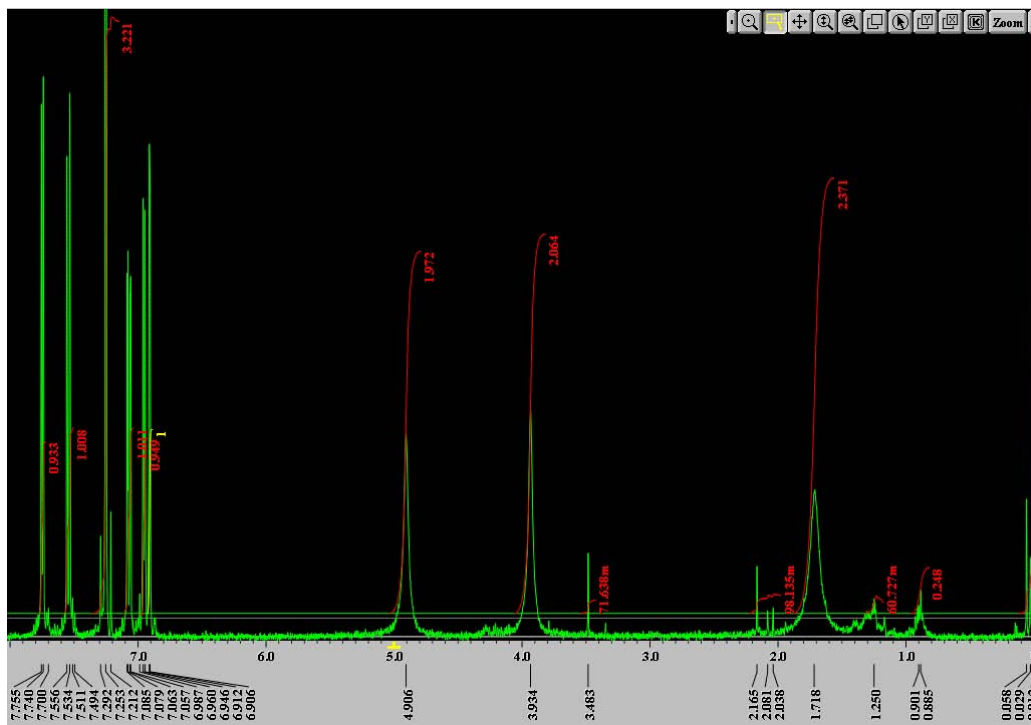


Figure 24 – ^1H NMR Spectra of Compound 7.

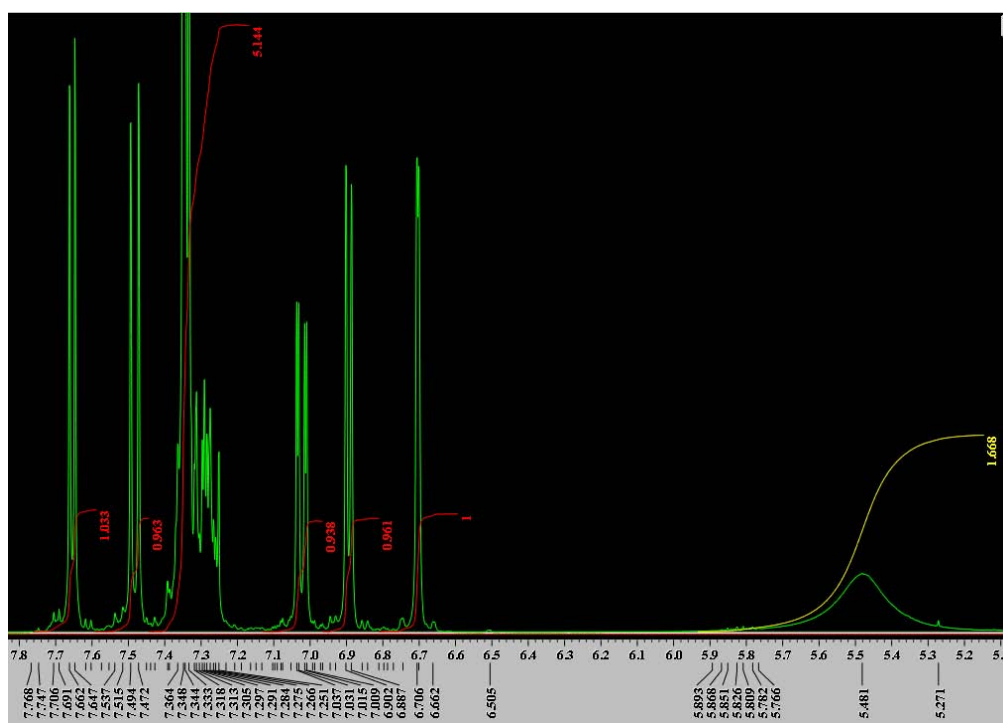


Figure 25 – ^1H NMR Spectra of Target 1.

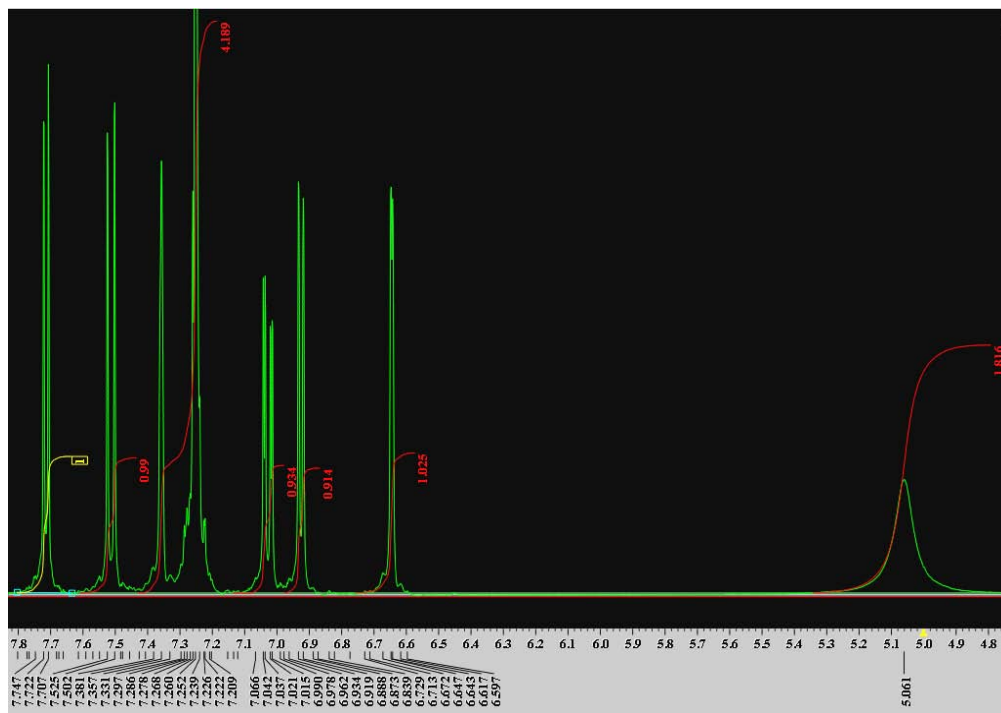


Figure 26 – ^1H NMR Spectra of Target 2.

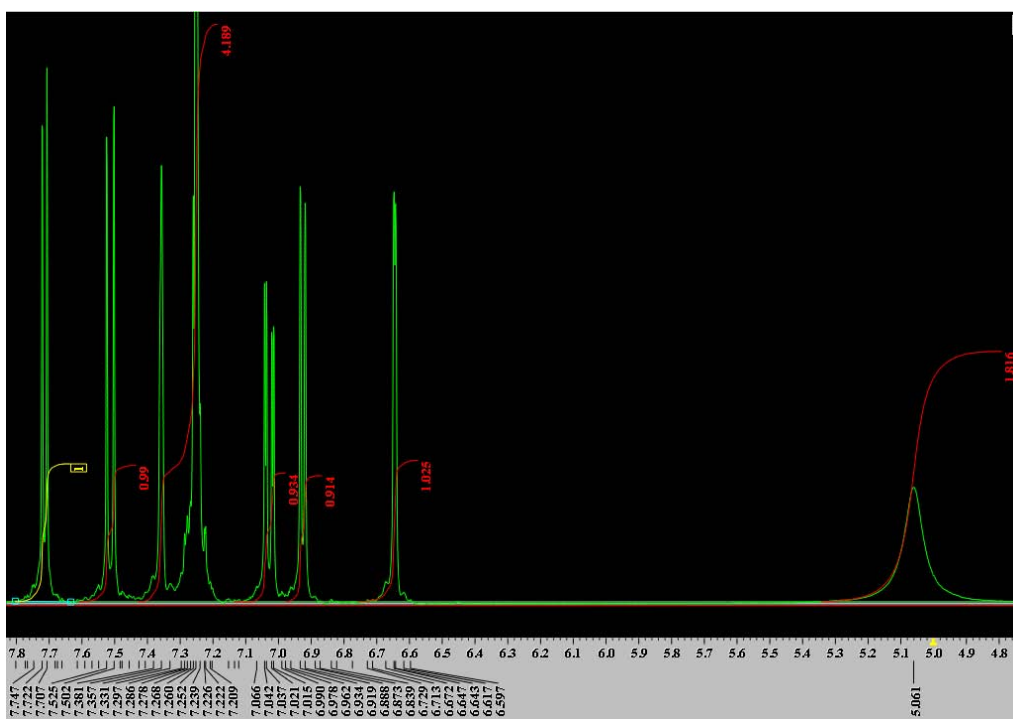
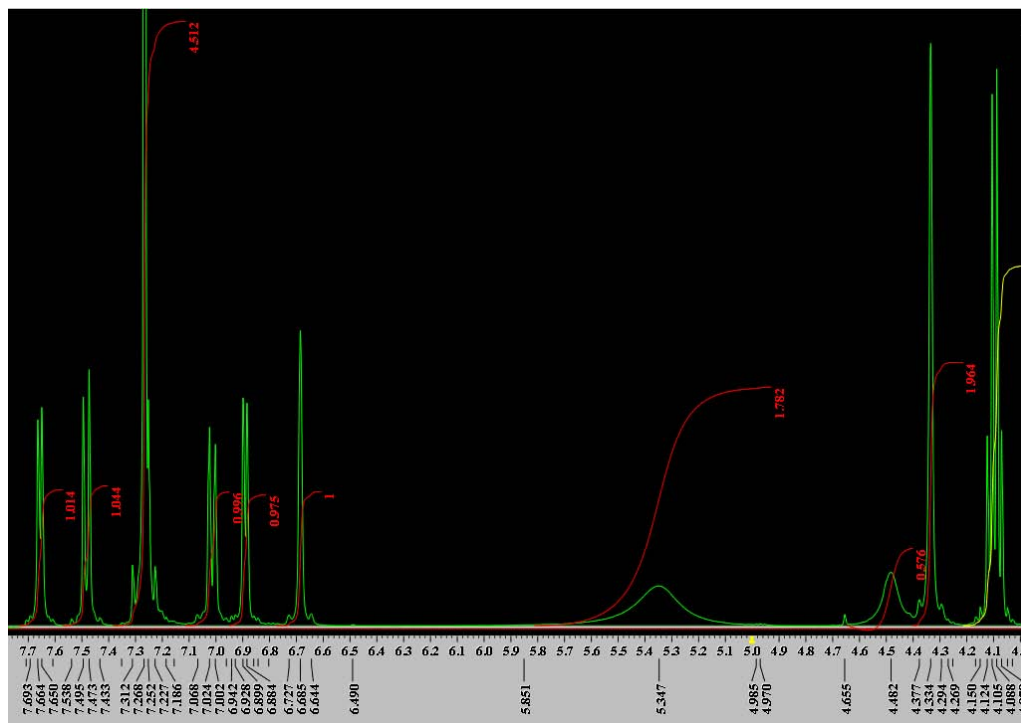
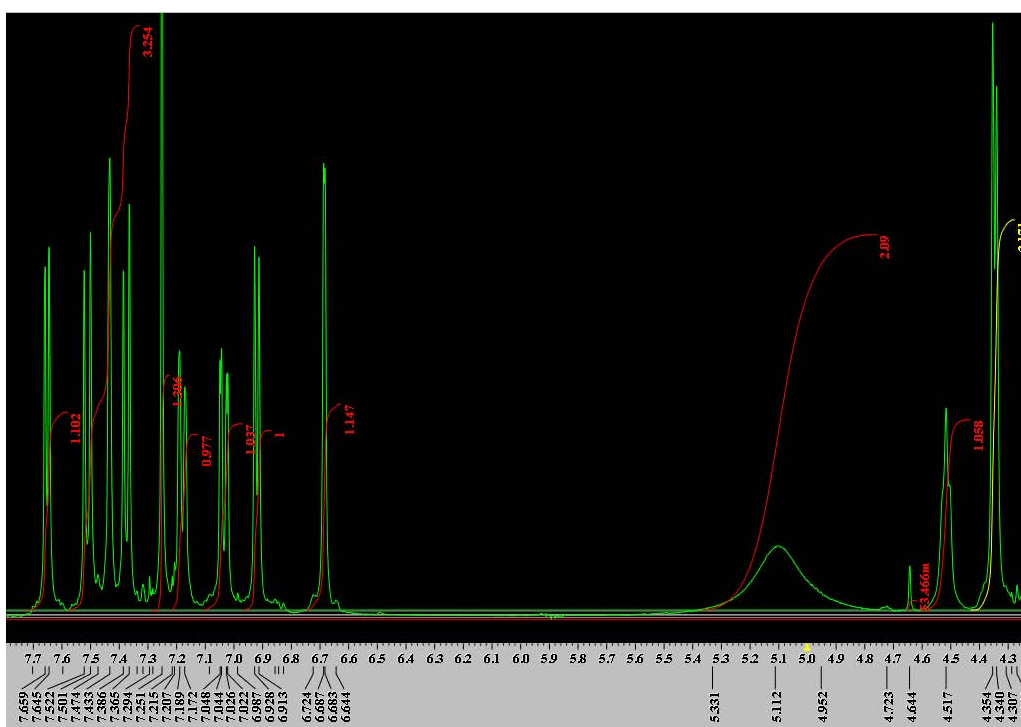


Figure 27 – ^1H NMR Spectra of Target 3.

Figure 28 – ¹H NMR Spectra of Target 4.Figure 29 – ¹H NMR Spectra of Target 5.

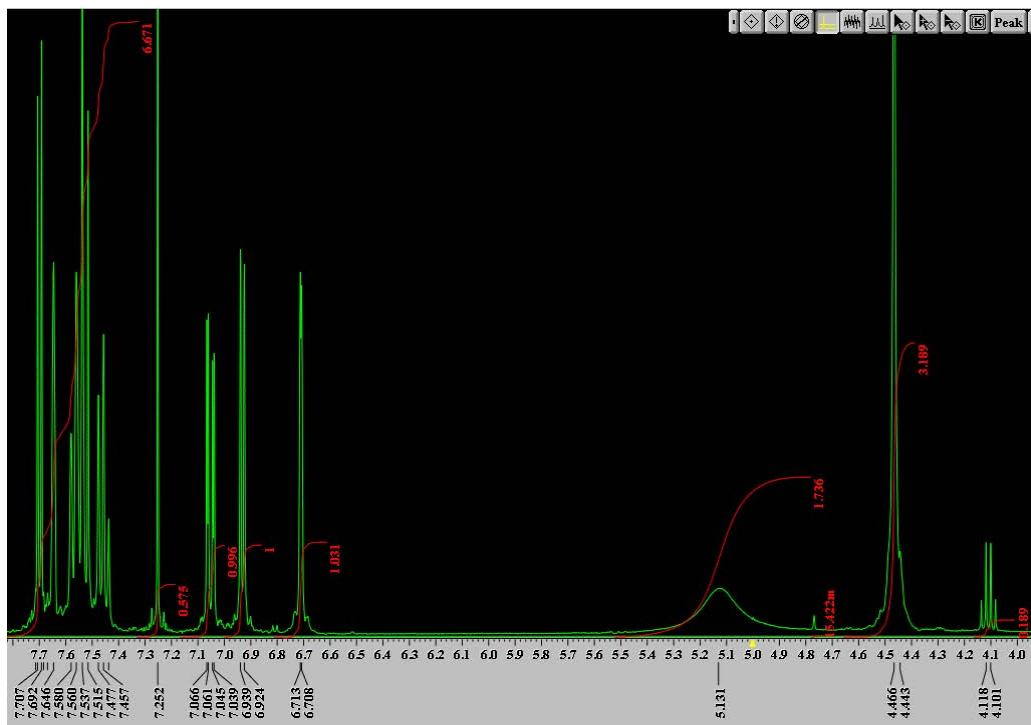


Figure 30 – ¹H NMR Spectra of Target 6.

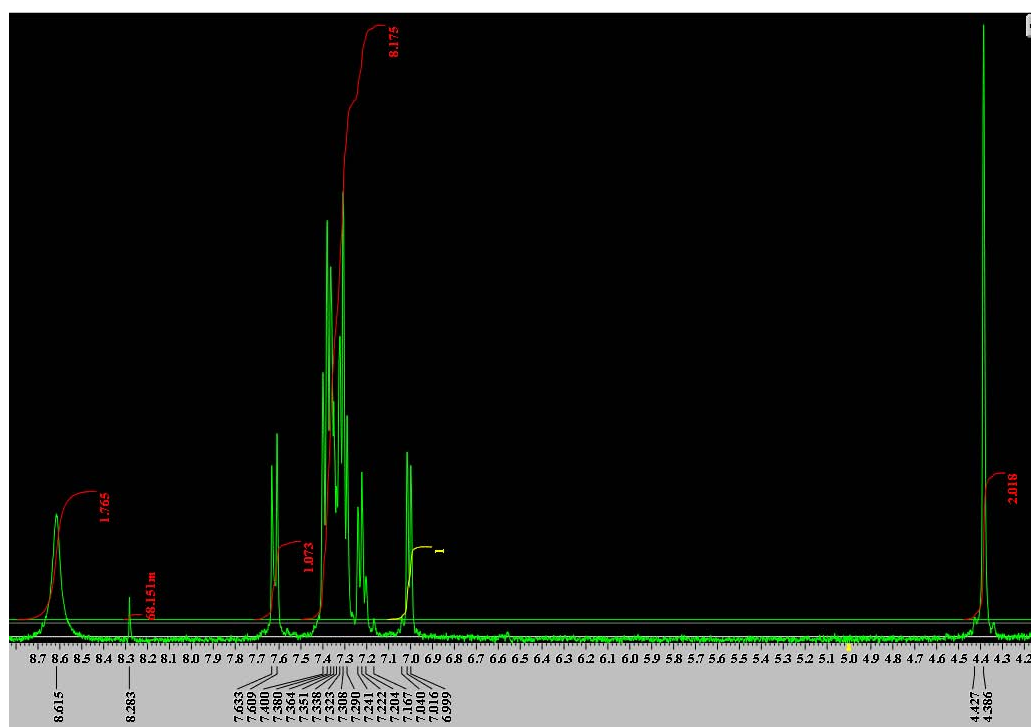


Figure 31 – ¹H NMR Spectra of Target 1 HCl Salt.

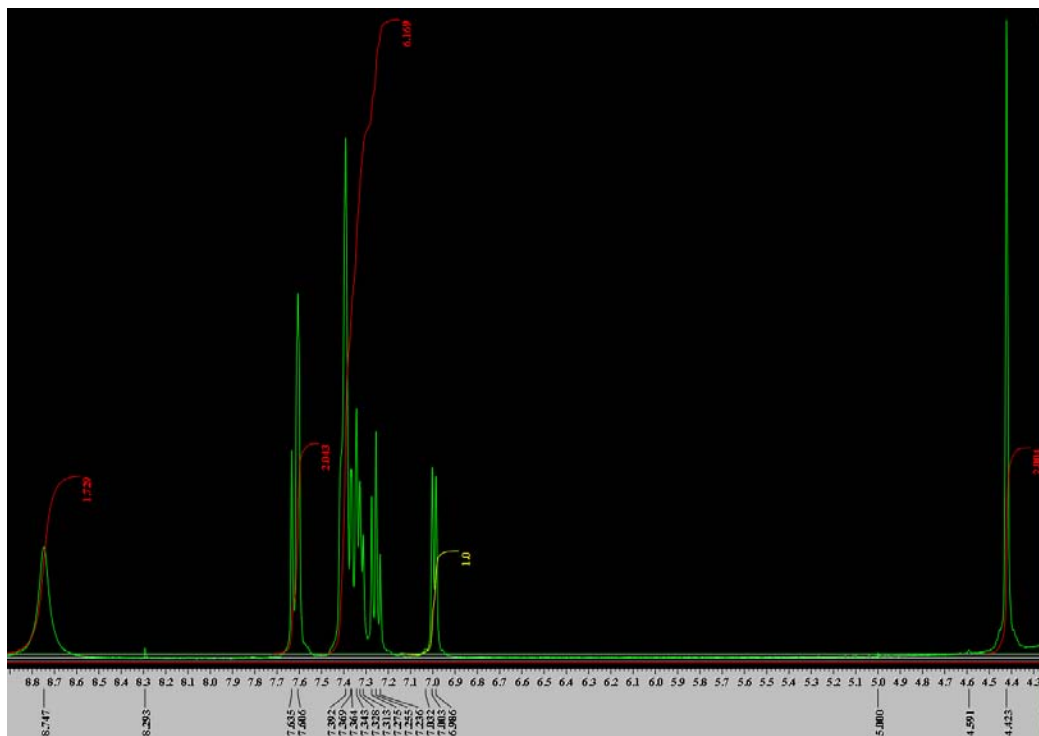


Figure 32 – ¹H NMR Spectra of Target 2 HCl Salt.

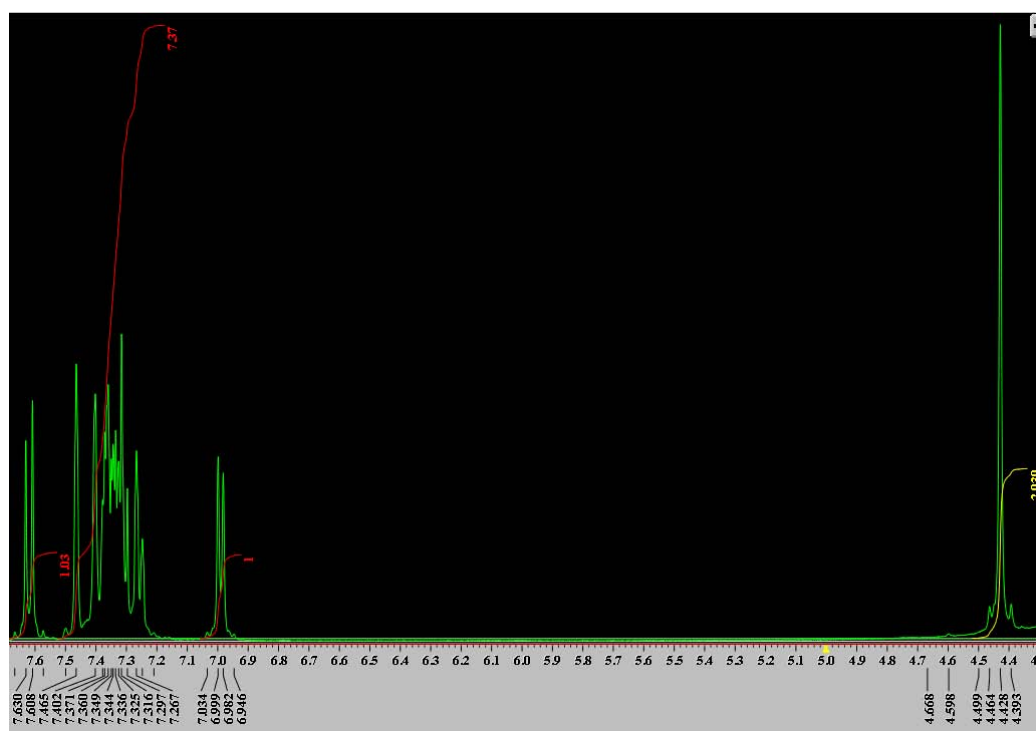


Figure 33 – ¹H NMR Spectra of Target 3 HCl Salt.

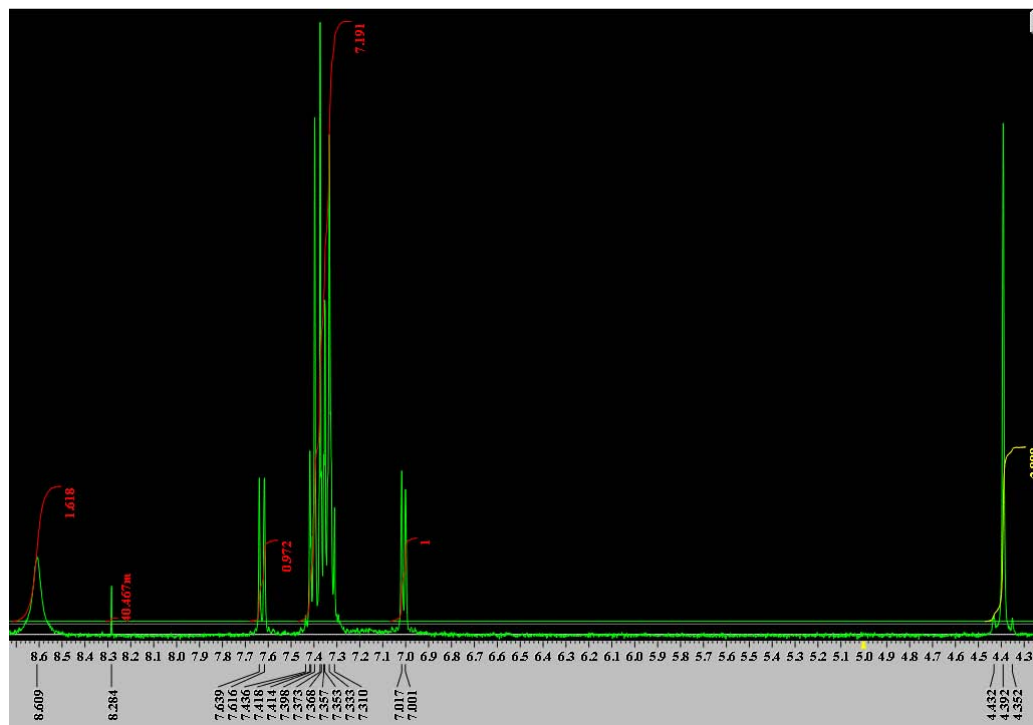


Figure 34 – ¹H NMR Spectra of Target 4 HCl Salt.

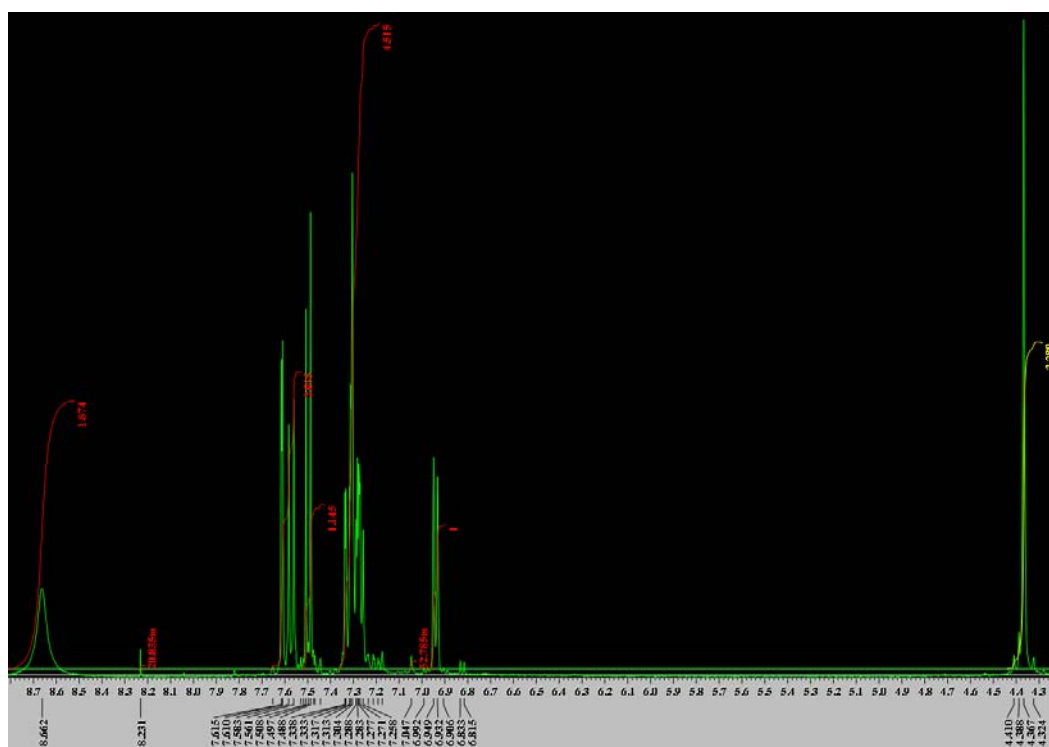


Figure 35 – ¹H NMR Spectra of Target 5 HCl Salt.

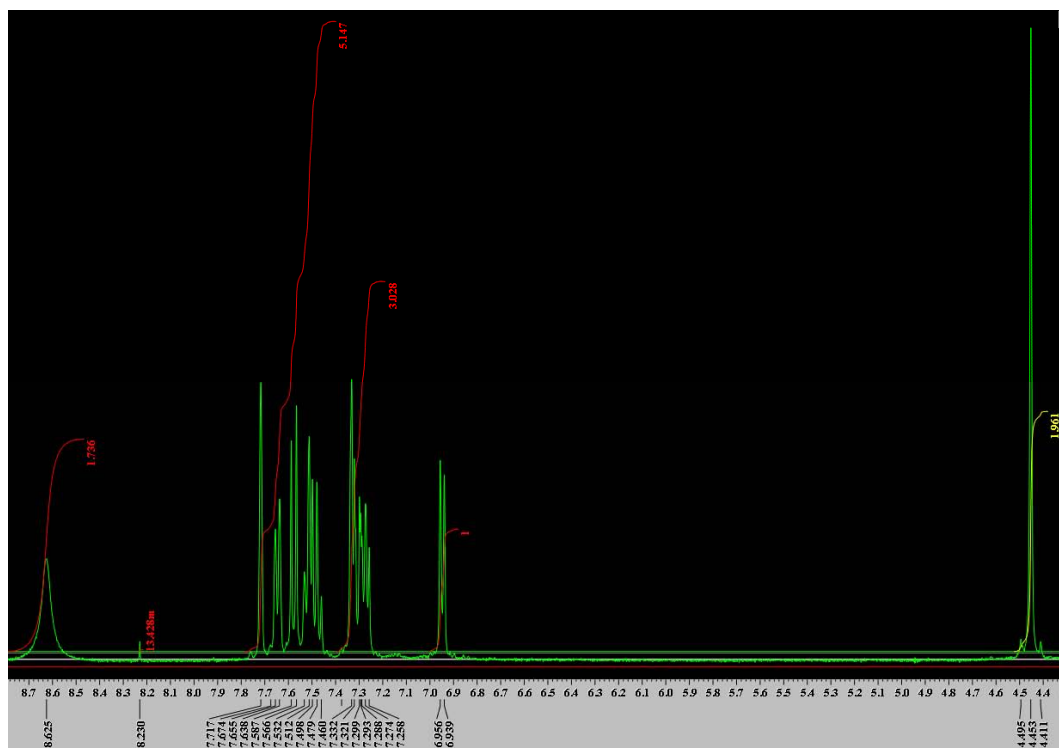


Figure 36 – ^1H NMR Spectra of Target 6 HCl Salt.